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Osmohomeostasis and Vacuole Biogenesis Genes in the Yeast *Saccharomyces cerevisiae*

by

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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To my wife Debby,

whose love, understanding,

and devotion are phenomenal!

DECLARATION

No part of this thesis has been previously submitted for any other degree in this or any other university. All work was performed by the author, except where otherwise stated.

Abstract

Martin Latterich, B.Sc. (Dunelm)

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A thesis submitted for a Ph.D., 1992.

The putative role of the *S. cerevisiae* vacuole in osmohomeostasis, as well as its biogenesis was analysed by taking a mutational approach. 97 mutants unable to tolerate high concentrations of salt were isolated and examined for aberrant vacuolar phenotypes. A comprehensive phenotypic analysis was able to demonstrate that apart from osmosensitivity most mutations conferred other properties such as altered vacuolar morphology, the inability to perform gluconeogenesis and/or the mislocalization of vacuolar proteins to the cell surface. The mutants fall into at least 20 complementation groups, termed *ssv* for *s*alt *s*ensitive *v*acuolar mutants, of which 3 genetically overlap with complementation groups isolated by others. This analysis provides evidence that in *S. cerevisiae* correct vacuolar biogenesis is required for osmotolerance as well as other important cellular processes. To elucidate vacuolar osmohomeostasis at the molecular level, one gene, *SSV7*, was cloned from a genomic DNA library by complementation of a *ssv7*-1 mutation and its sequence determined. It encodes a novel 927 amino acid protein with limited structural homology to the functional domains of two nucleotide exchange factors from *S. cerevisiae*, namely *CDC25* and *BUD5*. A mutation in the *SSV7* gene confers a pleiotropic phenotype including fragmented vacuoles and the absence of a nucleus. The mutation apparently uncouples mitosis from cell growth, which results in an increased cell size of a *ssv7*-1 mutant. The role of *SSV7* in the general concept of vacuole biogenesis is discussed, as is the observed fact that a number of mutants in intermediate biosynthetic pathways confer a vacuolar protein sorting defect.

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ABBREVIATIONS

All abbreviations other than those recommended by the IUPAC-IUB (Biochemical Nomenclature and related Documents, 1978) were avoided when possible. However, some abbreviations for frequently used terms for enzymes and some chemicals were thought to be an aid to the reader and are listed below. Other abbreviations for biological terms are defined at their first occurrance in the text.

API	Aminopeptidase I
APCo	Aminopeptidase Co
BCIP	Bromochloroindolyl phosphate
BSA	Bovine serum albumin
CDCFDA	5(6)-carboxy-2',7'-dichlorofluorescein diacetate
CIP	Calf intestinal phosphatase
CPY	Carboxypeptidase Y
CPS	Carboxypeptidase S
DAPI	4',6-diamino-2-phenylindole
dH_2O	Distilled water
DPAP A	Dipeptidyl aminopeptidase A
DPAP B	Dipeptidyl aminopeptidase B
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol

FITC	Fluorescein isothiocyanate
GlcNAc	N-Acetylglucosamine
IPTG	Isopropylthiogalactopyranoside
NBT	Nitro blue tetrazolium
OD_{600}	Optical density (absorbance) at a wavelength of 600 nm
P_i	Inorganic phosphate
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PrA	Proteinase A
PrB	Proteinase B
RT	Room temperature (20°C)
TE	10 mM Tris pH 8.0, 1 mM EDTA buffer
Tris	Tris-(hydroxymethyl)-methylamine-[2-amino-2-(hydroxymethyl)-propane-1,3-diol (tris)]
YMM	Yeast minimal medium

MEMORANDUM

The following publications have been achieved as a result of this research:

Latterich, M. and Watson, M.D. (1991) Isolation and characterization of osmosensitive vacuolar mutants in *S. cerevisiae*. *Mol. Microbiol.* **5**, 2417-2426.

Latterich, M., Hartley, C.A. and Watson, M.D. (1992) The plasticity of vacuole biogenesis in the yeast *Saccharomyces cerevisiae*. *J. Exp. Botany*, in press.

Latterich, M., Hartley, C.A. and Watson, M.D. (1992) A mutation in *SSV7*, a *CDC25* homologue, confers a viable anuclear phenotype. Manuscript in peparation.

Latterich, M. and Watson, M.D. (1992) A dual osmoregulatory mechanism in *S. cerevisiae*. Manuscript in preparation.

Latterich, M. and Watson, M.D. (1992) Osmoregulation in the yeast *Saccharomyces cerevisiae* (Review). Manuscript in preparation.

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CHAPTER 1

INTRODUCTION

1.1. General Introduction

One of the features that distinguishes eukaryotic cells from prokaryotes is that in eukaryotes many cellular processes are compartmentalized in distinct membrane bound organelles. These organelles are composed of a set of unique proteins, most of which are synthesized in the cytoplasm and co- or post-translationally targeted to the correct subcellular locus. This process is highly specific and requires specialized targeting mechanisms and signals which have to discriminate between different destinations or in case of the secretory pathway confer retention (Warren, 1987). A great deal of interest has focussed upon unravelling the targeting and sorting signals of proteins that are localized to organelles or secreted from the cell, and understanding the sorting mechanism. Another area of interest is the assembly, maintenance and partitioning of these organelles during the cell cycle. Daughter buds must inherit a minimal number of all organelles and cytoplasmic constituents necessary for independent survival (Yaffe, 1991). The segregation of organelles has to precede a phase of protein and lipid biosynthesis to provide sufficient material for segregation to occur. Except mitochondria and chloroplasts, which possess their own genome and are capable of synthesising part of their protein constituents autonomously, other organelles, such as the nucleus, the endoplasmic reticulum, the Golgi apparatus, the lysosome in animal cells, or the vacuole in yeast and plants derive all their protein constituents from the cytoplasm. The biogenesis of the Golgi apparatus, the vacuole and the plasma membrane all depend upon delivery of proteins via branches of the secretory pathway. Thus the majority of their constituents have to be translocated into the ER and transported to subsequent locations.

The vacuole of the yeast *Saccharomyces cerevisiae* has become a recent focus of investigation, partly because of its comparative homology to mammalian lysosomes and its easy amenability to genetic and biochemical analysis (Achstetter and Wolf, 1985; Klionsky *et al.*, 1990; Raymond *et al.*, 1992). It represents a prominent organelle which participates in a series of important cellular processes, as diverse as proteolytic processing and degradation of vacuolar as well as cytoplasmic proteins (Achstetter and Wolf, 1985; Chiang and Schekman, 1991), nitrogen metabolism (Jones, 1982), amino acid, carbohydrate and ion storage (Klionsky *et al.*, 1990), sporulation (Teichert *et al.*, 1989) and ion and osmohomeostasis (Klionsky *et al.*, 1990). While many studies have addressed particular aspects of the vacuolar function *in vivo* and *in vitro*, its biogenesis is still poorly understood. The present study intends to examine two related aspects of its biogenesis, namely is morphological integrity and its role in osmoregulation, of which the latter has been hypothesized to be essential to allow survival under unfavourable environmental conditions (Banta *et al.*, 1988; Klionsky *et al.*, 1990).

In the following introduction some general features of organelle biogenesis are reviewed to allow a comparison between the biogenesis of the vacuole with that of other organelles. Numerous studies in the past decade have focussed on various aspects of organelle inheritance and biogenesis in different organisms, and a complete review of the literature would easily exceed the number of pages of this thesis several-fold. Therefore, only the most important features are mentioned. Also, since many mechanisms of protein targeting and organelle biogenesis are conserved across species, it was decided to concentrate describing these processes in *Saccharomyces cerevisiae*, lastly because this simple model system has contributed to the undestanding of many cellular processes due to its biochemical as well as genetical amenability. However, when basic features differ from mammalian and plant systems they are briefly outlined to allow a distinction. Protein sorting in plants has not received as much attention as in mammalian cells or yeasts. Recent studies show that the sorting machinery is conserved between plants, yeasts and mammalian cells. Therefore it was decided that unless plants have unique features in organelle biogenesis, other comparatative features such as protein import into the ER (Iturriaga *et al.*, 1989), Golgi processing and morphology

(Moore *et al.*, 1991a), vacuolar protein sorting (Sturm *et al.*, 1988) and endocytosis (Horn *et al.*, 1992) will not be mentioned. They are outlined in some excellent reviews, mostly by Maarten J. Chrispeels and collaborators (Chrispeels, 1991; Chrispeels and Raikhel, 1992; Chrispeels and Tague, 1991; Vitale and Chrispeels, 1992; Harris and Watson, 1991).

1.2. Protein Import into the Nucleus

One of the most obvious distinctive features that discriminates eukaryotes from prokaryotes is the nucleus. The nucleus represents a double bilayer membrane enclosed compartment in which chromosomes and the ribosome assembly take place separated from the remaining cytoplasm. During mitosis the *S. cerevisiae* nucleus elongates, and part of it migrates into the newly formed bud. The morphological changes during mitosis differ from that in other eukaryotes in that it remains intact during the whole cell cycle (closed mitosis), whereas it fragments or vesicularizes in other eukaryotes. The nucleus, ER and Golgi complex disassemble during mitosis in mammalian cells (Fry, 1976; Zeligs and Wollman, 1979; Lucocq *et al.*, 1987) and membranes derived from these organelles are dispersed through the cytoplasm until the end of mitosis to ensure partitioning of the organelles during cell division. At the end of mitosis these membraneous compartments reassemble by fusion, a process which might be regulated by the same factors that mediate vesicle budding and fusion in interphase cells (Warren, 1985). Nuclear vesicles will bind to chromatin, and after a step involving GTP hydrolysis will fuse to form a nuclear envelope in the presence of cytoplasm (Boman *et al.*, 1992; Newport and Dunphy, 1992). The assembly of the nuclear membrane is independent of the availability of lamins (Newport *et al.*, 1990). Karyogamy (nuclear fusion) after successful mating occurs and preceeds the formation of diploid *S. cerevisiae* (Rose, 1991). During mating cells of opposite mating type exchange peptide mating pheromones. Responding cells arrest in G1 of the cell cycle and develop cellular projections termed shmoos, after which cell walls dissolutions and plasma membrane connections allow the cells to fuse and form the zygote. The haploid nuclei associate by

interdigitating cytoplasmic microtubules arising from the two spindle pole bodies present in the nuclear envelope and fuse together by an unknown mechanism in proximity of the spindle pole body.

The nuclear membrane is stabilized by the karyoskeleton, the nuclear lamina, a network of intermediate filaments which attach to the intranuclear membrane and are thought to be associated with chromatin. Much knowledge about the karyoskeleton comes from amphibian and mammalian systems, where different lamins have been identified (Krohne *et al.*, 1987). While lamin B is constitutively expressed and present in all cells, lamin A and C are differentially expressed. It has been proposed that lamin A participates in early differentiation by reorganizing chromatin that is followed by expression of a differential phenotype (Collard *et al.*, 1992; Wolin *et al.*, 1987). The search for evolutionary conserved yeast lamins so far has been unsuccessful (M.N. Hall and K. Weber to M.D. Watson, personal communication) despite one initial report that lamin A, lamin B and a lamin B receptor had been identified in yeast based on immunological cross reactivity (Georgatos *et al.*, 1989). A gene showing sequence homology to the lamin B receptor has recently been identified (Shimanuki *et al.*, 1992). The apparent lack of evolutionary conserved lamin homologues can be explained because yeast does not differentiate. Other structurally similar intermediate filament-like proteins such as Nuf1p have been identified which could be part of the karyoskeleton (Mirzayan *et al.*, 1992). Lamins A and C are not essential proteins in mammals since cell lines lacking both lamins remain viable (Guilly *et al.*, 1987). The nuclear membrane possesses pore complexes (125,000 kDa) to facilitate the selective uptake of cytoplasmic proteins. Its structure is conserved between mammalian species and yeast (Aris and Blobel, 1989; Goldfarb and Michaud, 1991; Wozniak *et al.*, 1989). Nuclear specific import is determined by nuclear localization sequences, which are characterized by small positively charged stretches of amino acids that are not proteolytically removed (Dingwall and Laskey, 1986). While the nuclear localization signals do not have a common consensus, there can be more than one signal in a nuclear protein. The yeast repressor $\alpha 2$ contains two putative nuclear localization signals, of which one is important in nuclear import, while the other appears to facilitate binding to

the nuclear pore complex (Hall *et al.*, 1990). Nuclear uptake of proteins is highly selective (Dingwall and Laskey, 1986). The size of proteins imported varies from small histones to the large (2800 kDa) 60S ribosomal subunit which requires a mechanism of dilation of the relatively small (9-11 nm diameter) nuclear pore (Goldfarb and Michaud, 1991).

Nuclear import in mammalian cells requires soluble cytoplasmic factors, ATP and lastly the presence of a nuclear localization signal in the protein to be imported into the nucleus (Adam *et al.*, 1990; Halleck and Rechsteiner, 1990). In the absence of ATP or at low temperature nuclear proteins were localized to the nuclear membrane but failed to be imported. An N-ethylmaleimide-sensitive cytosolic factor is required in signal mediated binding to the nuclear pore (Newmeyer and Forbes, 1990). Nucleoplasmin, a nuclear protein, complexed with gold particles will bind to eight distinct features to the nuclear pore complex around the periphery of the transporter and also over the center of the nuclear pore complex (Akey and Goldfarb, 1989). The nucleoplasmin-gold particles are then translocated through the nuclear pore into the nucleus. Proteins that have entered the nucleus are generally accumulated against a concentration gradient and retained in the nucleus and cannot easily diffuse out, suggesting the existence of a separate retention mechanism (Dingwall and Laskey, 1991). The transport efficiency of a given nuclear protein depends on the number of nuclear localization signals present (Dworetzky *et al.*, 1988). There is evidence that a number of nuclear localization signal binding proteins associate with proteins and shuttle between the cytoplasm and nucleolus (Meier and Blobel, 1990; Li and Thomas, 1989; Silver *et al.*, 1989; Stochaj and Silver, 1992). Spliced mRNA is exported from the nucleus, probably also through a nuclear pore by an unknown mechanism, which has to control the selective export of spliced but not unspliced mRNA (Goldfarb and Michaud, 1991). Also, nuclear U snRNAs are exported from and imported into nuclei via the nuclear pore complex by a mechanism that requires a different receptor to that of nuclear proteins (Michaud and Goldfarb, 1992; Goldfarb and Michaud, 1991). A DnaJ protein homologue, *YDJ1*, has been reported to be localized in a perinuclear fashion as well as in the cytoplasm and associated with the ER. A deletion of the gene confers a conditional lethal phenotype

(Caplan and Douglas, 1991), suggesting that it is required for some essential cellular function.

A number of proteins have to enter the nucleus during the cell cycle in a regulated fashion such as the tanscription factor SWI5 which is accumulated in the cytoplasm and is then taken up during the G1 phase of the cell cycle (Henkel *et al.*, 1992; Nasmyth *et al.*, 1990). Whether this factor is degraded in the nucleus or exported to a cytoplasmic location is not clear.

1.3. Protein Import into Mitochondria

Although mitochondria have their own genome and protein synthesis machinery, the biogenesis of the organelle relies on the import on nuclear encoded proteins. Very early in the cell cycle a portion of a mitochondrion migrates into the developing bud, a process dependent on the presence of actively growing mitochondria and possible cytoskeletal elements (Yaffe, 1991). Many mitochondrial proteins are synthesized as larger precursors with transient N-terminal peptide extensions (Geli and Glick, 1990; Glick and Schatz, 1991; Neupert *et al.*, 1990). These extensions contain several basic amino acids, lack acidic amino acids and are processed during or after import. The β-subunit of the mitochondrial F_1-ATPase has a 27 amino acid N-terminally located sequence which is necessary and sufficient for import into the organelle (Emr *et al.*, 1986). Although mitochondrial signal peptides have no common consensus, the signal cleavage site is predictable and often represents RXXS/A (Gavel and von Heijne, 1990). As little as 12 N-terminal amino acids suffice to target fusion peptides to the mitochondrion (Hurt *et al.*, 1985). However, the pre-sequence processing is impeded, which indicates that further sequence information is necessary for correct proteolytic processing to occur. Different mitochondrial proteins are imported via a common translocator present on "contact sites" of outer with inner membrane (Pfaller *et al.*, 1988; Pon *et al.*, 1989; Roise, 1992) although different import receptors exist for two subclasses of mitochondrial proteins in a common receptor complex (Geli and Glick, 1990; Söllner *et al.*, 1990; Söllner *et al.*, 1992; Stuart *et al.*, 1990). In case of

apocytochrome c, the precursor form of cytochrome c, there is no receptor/translocator present on the outer mitochondrion membrane and insertion into the membrane is spontaneous (Stuart *et al.*, 1990). The receptor complex in the outer membrane consists of several preprotein receptors, Isp49p and Mas70p (*S. cerevisiae*) or MOM72 and MOM19 (*N. crassa*), close to the insertion site in conjunction with low molecular weight proteins (Isp42, MOM38, MOM30, MOM22, MOM8 and MOM7). The binding of preproteins to MOM72 occurs outside of the complex, upon which it associates with the translocation complex. Preproteins accumulating at the import site are partially exposed to the intermembrane space. Subsequent translocation across or into the inner membrane requires a membrane potential $\Delta\psi$ and the *mhsp*70 protein in the matrix. It has been suggested that the positively charged amino acid residues respond to the membrane potential and are transported across the inner membrane upon which proteolytic processing of the import sequence occurs (Neupert *et al.*, 1990). The proteolytic processing is mediated by the *MAS1* and *MAS2* gene products, two soluble matrix proteins (Yaffe *et al.*, 1985; Geli and Glick, 1990). These form a protein, called MpI, of approximately 115 kDa (Achstetter and Wolf, 1985). The protein import across the mitochondrial membrane is facilitated by sequential action of two heat shock related proteins, *mhsp*70 and *hsp*60 (Glick and Schatz, 1991; Manning-Krieg *et al.*, 1991; Scherer *et al.*, 1990) and also depends on the conformation of the protein to be translocated because unfolding prior to translocation is essential for successful translocation to occur (Nye and Scarpulla, 1990a; Nye and Scarpulla, 1990b; Rassow *et al.*, 1989; Vestweber and Schatz, 1988). The *mhsp*70 chaperone probably plays an important part in the translocation process and after ATP dependent release of the imported protein *hsp*60 mediates correct folding of the protein, also connected with hydrolysis of ATP. Some of the proteins participating in the import of nuclear encoded proteins into mitochondria are encoded by the mitochondrial genome (Myers *et al.*, 1985).

Some mitochondrial proteins destined for the inter-membrane space enters mitochondria by different mechanisms. Cytochrome c enters the mitochondrium independent of a translocation apparatus (Glick and Schatz, 1991). Other proteins have

a bipartite pre-sequence containing an amino terminal matrix targeting signal, followed by a stretch of hydrophobic amino acids. The mechanism by which the proteins enter first the matrix and processed to be released into the intermembrane space is not clear. Proteins are either completely localized into the matrix and after signal peptide cleavage are then exported mediated by their hydrophobic pro-sequence. Alternatively, the hydrophobic sequence could act as a stop-transfer sequence during translocation, and proteolytic cleavage in the inner membrane space could release the protein. Inter-membrane space proteins undergo two proteolytic events. The first is accomplished by the matrix protease (Böhni *et al.*, 1983), the second by the *PET2858* gene product, *IMP1*, which shows sequence similarity to the *E. coli* leader peptidase and might be evolutionally conserved (Behrens *et al.*, 1991). Targeting of proteins to the inner membrane is more complex and might involve stop-transfer signals (Glick and Schatz, 1991). The assembly of outer membrane proteins is at present unknown and does not involve obvious mitochondrial signal sequences but requires specific receptors (Geli and Glick, 1990; Glick and Schatz, 1991).

1.4. Protein Import into Chloroplasts

Chloroplasts are organelles which multiply by fission after replication of their genomes. Chloroplasts interact with microfilaments and are subject to movement along these filaments depending on light conditions (Haupt and Scheuerlein, 1990). Chloroplasts, like mitochondria, import a large number of proteins, although they have their own genome and protein synthesis machinery. The chloroplast is surrounded by a two membrane envelope separated by an intermembrane space except for "contact points" where membranes are fused or in close proximity (DeBoer and Weisbeek, 1991). The inner membrane surrounds the stroma where CO_2 fixation and most biosynthetic pathways occur. The stroma also contains thylakoids which are membranes participtating in the photosynthetic electron transfer and surround the thylakoid lumen where water splitting and oxygen evolution take place. The thylakoid lumen is highly acidic due to the H^+ pumping activity during photosynthesis. Nuclear encoded

chloroplastic proteins are synthesized cytoplasmically as precursors with a N-terminal extension. This transit peptide contains sufficient and necessary information for import into the chloroplast (Keegstra and Olsen, 1989; von Heijne *et al.*, 1989). Transit peptides are characterized by high amounts of hydroxy amino acids and the lack of tyrosine and acidic amino acids (DeBoer and Weisbeek, 1991). A typical transit peptide represents an amino-terminal domain of 10 amino acids with no proline, glycine or charged amino acids followed by a central region with high amounts of hydroxy amino acids and containing positively charged amino acids, and lastly a C-terminal 10 amino acids with a predicted β-sheet forming capacity. Proteins destined for the stroma only require the transit peptide for correct import and localization (Hand *et al.*, 1989). The targeting information of proteins to be imported into the thylakoid lumen is present in the form of a bipartite transit peptide, a transit domain for import into the chloroplast and a separate domain destining the peptide for import into the thylakoid (Ko and Cashmore, 1989). Analysis of protein import of plastocyanin (PC), a chloroplast lumenal thylakoid protein, revealed that transport occurs in two steps: import across the chloroplast envelope into the stroma, followed by transfer across the thylakoid membrane into the lumen (Hageman *et al.*, 1990). The N-terminal peptide of PC is removed by two separate processing proteases, the first which removes the chloroplast import signal and the second which removes the thylakoid routing signal. The thylakoid targeting sequence is short and similar to a bacterial signal sequence (DeBoer and Weisbeek, 1991). It contains a few positively charged N-terminal amino acids, a central hydrophobic domain then a region containing small uncharged amino acids in -3 and -1 positions relative to the protease processing site.

Protein transport into or across the thylakoid membrane is protein specific and depends solely on the presence of a trans-thylakoid ΔpH for some proteins (OE17, OE23), or dependent on ATP and ΔpH (OE33) or ATP only (PC) (Cline *et al.*, 1992). The study of the protein targeting step with gene fusions *in vitro* is complicated by the fact that proper localization only occurs *in vivo* (DeBoer *et al.*, 1991) which questions some of the experimental results on thylakoid import in earlier studies. Proteins located in the thylakoid membrane exhibit the same charge assymmetry as other transmembrane

domain proteins and generally have the more highly charged regions facing the stromal site (Gavel *et al.*, 1991). Whether the chloroplast chaperonin 60 functions in unfolding of proteins before import into the thylakoids is not known (Lubben *et al.*, 1989). The precise import apparatus required for the recognition of chloroplasmic enzymes and translocation across into the stroma and sometimes thylakoid has not been unambiguously identifed and is thought to be similar to mitochondrial import of proteins (de Boer and Weisbeek, 1991). The chloroplast import receptor has been identified as an integral membrane protein (Schnell *et al.*, 1990). It also appears that different proteins are imported by different mechanisms.

1.5. Protein Import into Peroxysomes

The peroxysome, glyoxysome, glycosome and glucosome (microbodies) are small related organelles bound by a single membrane which undergo fission or budding to multiply. Peroxysomes carry out different metabolic functions that grossly depend on cell type and metabolic state of the cell, including catalase dependent reactions, β-oxidation of fatty acids, the glyoxalate cycle, alcohol oxidation, transaminations and purine and polyamine catabolism (Lazarow and Fujiki, 1985). Proliferation of the organelle is inducible during certain growth conditions (Erdmann *et al.*, 1991), and proliferation occurs by posttranslational import of proteins which divide and form daughter peroxysomes. The origin of the membrane is not known, but is probably not derived from the endoplasmic reticulum as has been suggested before (Trelease, 1984). Peroxysomes are probably degraded in the vacuole (H.L. Chiang, personal communication). Peroxysomal proteins are synthesized on free polysomes and most are imported posttranslationally not involving any obvious modification (Lazarow and Fujiki, 1985; Subramani, 1992). The mechanism of translocation is conserved between yeast, plants, insects and mammals and in different microbodies providing evidence for a common evolutionary conservation between species and classes of microbodies (Gould *et al.*, 1990; Keller *et al.*, 1991; Subramani, 1992). Most peroxysomal proteins in insects have the C-terminal tripeptide SKL (Gould *et al.*, 1988; Gould *et al.*, 1989)

which is also conserved in mammalian cells (Gould *et al.*, 1990a; Gould *et al.*, 1990b). Zellweger cell lines from patients with a peroxysomal disorder are unable to import proteins with the SKL targeting signal caused by mutations in different complementation groups (Santos *et al.*, 1992; Walton *et al.*, 1992). Another class of peroxysomal proteins do not have the C-terminal SKL tripeptide and are not imported into peroxysomes by the same mechanism (Kamijo *et al.*, 1992). 3-ketoacyl-CoA thiolase has a N-terminal peroxysomal import signal which is cleaved upon translocation into the peroxysome, arguing for distict import pathways (Swinkels *et al.*, 1991). Two mutants have been isolated which are deficient in the assembly of peroxysomes (Tsukamoto *et al.*, 1990).

1.6. The Secretory Pathway

The secretory pathway in *S. cerevisiae* has been defined by the isolation and characterization of conditionally lethal mutations in which protein transport is arrested intracellularly because surface growth and secretion are linked (Deshaies and Schekman, 1987; Ferro-Novick *et al.*, 1984; Holcomb *et al.*, 1988; Novick and Schekman, 1979; Novick and Schekman, 1983; Novick *et al.*, 1980; Schekman *et al.*, 1983). Biochemical and morphological examination of the *sec* (*sec*retory) mutants allowed the classification of the compartments that were affected by each mutation and to establish the order of events in secretion (Esmon *et al.*, 1981; Ferro-Novick *et al.*, 1984; Novick *et al.*, 1981; Kaiser and Schekman, 1990). These pioneer studies established unequivocally the proposed secretory pathway from the ER to Golgi to the plasma membrane or vacuole. Subsequent studies demonstrated that all secretory and plasma membrane proteins travel via the same vesicular intermediates (Brada and Schekman, 1988; Holcomb *et al.*, 1988; Tschopp *et al.*, 1984).

Not all proteins exit the cell by migration through compartments of the secretory pathway (Muesch *et al.*, 1990). The a-mating factor is not imported into the ER but modified cytoplasmically by proteolytic processing and farnesylation and methylation of its C-terminal Cys residue (Schafer *et al.*, 1989). The peptide hormone exits the

cytoplasm via a putative protein translocation channel encoded by the *STE6* gene product (Kuchler *et al.*, 1989). A *ste6* mutant can be functionally complemented by a mammalian multidrug resistance gene which leads to a restoration of **a**-factor export (Raymond *et al.*, 1992). Recent evidence points towards a second late secretory pathway from the yeast Golgi apparatus in yeast which is different from the constitutive pathway (Lupashin *et al.*, 1992). A 400 kDa heat shock induced glycoprotein has been identified whose export to the plasma membrane is dependent on Sec18p but not on Sec7p. The protein also does not comigrate with the secretory acid phosphatase in the same secretory vesicles.

A number of heritable pathological defects are caused by perturbations in the secretory process, such as the mutation induced misfolding and subsequent retention of the type IIA von Willebrand factor involved in haemostasis (Lyons *et al.*, 1992) and the tissue dependent missorting of lysosomal proteins in I-cell disease resulting from a defective phosphotransferase (Kornfeld, 1986).

1.6.1. Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a network of interconnected membrane lamellae supported by microtubules (Lee *et al.*, 1989). The ER is divided into two subsets, the smooth ER and the rough ER where ribosome binding and protein transport occurs (Sanderson *et al.*, 1990; Sanderson and Meyer, 1991). The ER is the major site of membrane phospholipid biosynthesis, and ER membranes can proliferate in the presence of certain protein factors that presumably induce lipid biosynthesis (Schunck *et al.*, 1991; Wright *et al.*, 1988). The ER and nuclear envelope are connected to each other, a fact that probably ensures partitioning of the ER during mitosis (Yaffe, 1991). Protein import into the ER is a multistep process, which requires a number of cytosolic and ER membrane factors (Walter and Lingappa, 1986; Walter *et al.*, 1984). Proteins destined for the ER, analogous to nuclear encoded mitochondrial proteins, usually have a N-terminal signal peptide carrying a positive sorting sequence. Analysis of secretory routing signals of different proteins showed that a N-terminal hydrophobic region

preceeded by one to three positively charged amino acids contains the necessary information. Fusion of N-terminal signal peptides to cytoplasmic proteins showed that they were sufficient to cause targeting into the ER. The specificity of the pre-sequence appears to be low in yeast, since a significant amount of random sequences can act as a secretory pathway routing signal (Kaiser and Botstein, 1990). Carboxypeptidase Y and acid phosphatase can even be partially translocated without functional pre-sequence (Blachly-Dyson and Stevens, 1987; Haguenauer-Tsapis, 1992). In mammalian cells the functional efficiency of a signal peptide is related to its hydrophobicity (Bird *et al.*, 1990). For instance the yeast vacuolar protein carboxypeptidase Y can only be translocated into mammalian microsomes if one glycine is substituted by leucine which increases the hydrophobicity of the signal peptide. Functional signal peptides can adopt several structures: highly ordered before association with the signal recognition particle, or less ordered when in a lipid environment (Caulfield *et al.*, 1991). Complex membrane proteins having several transmembrane domains in *E. coli* can adapt a correct conformation in the absence of a N-terminal export signal (Ehrmann and Beckwith, 1991). Thus the first signal does not dominate the insertion process, and there are further putative topological signals promoting the assembly of surrounding amino acids. Proteins anchored in the ER membrane by an uncleaved signal peptide can assume two orientations. Type I signal anchor sequences translocate the N-terminus across the membrane, while type II signal anchors translocate the C-terminus, using the same translocation apparatus (High *et al.*, 1991a). The orientation of the protein in the membrane appears to be determined by the charge distribution. Signal anchoring sequences occur in the form of a tripartite structure: a hydrophobic core region that mediates targeting to and insertion into the ER membrane and flanking hydrophilic segments that determine the orientation of the protein in the membrane (Haeuptle *et al.*, 1989). Not all ER membrane associated proteins have N-terminal transmembrane anchors. The nontransmembrane tyrosine phosphatase PTP-1B can localize to the ER via its 35 C-terminal amino acids (Frangioni *et al.*, 1992).

The signal recognition particle (SRP) is an essential component for protein translocation (Meyer, 1991; Rapoport, 1991; Walter and Lingappa, 1986). It associates

with ribosomes to facilitate the recognition of signal sequences. Upon emergance of the signal peptide translocational arrest occurs until the ribosone/SRP/nascent protein complex is bound to the ER by sequential interaction with the SRP receptor (Meyer, 1991). The SRP receptor binds GTP to mediate the release of the signal peptide. Subsequent GTP hydrolysis enables the release of the SRP from the SRP receptor (Rapoport, 1990). A signal recognition particle is found in yeast. Several of its subunits have been identified (Amaya *et al.*, 1990; Hann and Walter, 1991; Stirling and Hewitt, 1992). The 54 kDa SRP subunit homologue from mammalian cells has been cloned from yeast (Amaya *et al.*, 1990; Hann *et al.*, 1989). It is a 59.6 kDa protein essential for efficient cell growth in most yeast strains, and is also highly homologous to the SecY protein in *E. coli*. SRP54 is responsible for both interaction with the signal peptide and binding to the RNA of the SRP (High and Dobberstein, 1991; Römisch *et al.*, 1990; Walter and Blobel, 1982). It has a GTP binding site, but whether or not GTP hydrolysis is associated with signal recognition or assembly of the SRP is not known. This is despite the GTP requirement for translocation in mammalian cells (High *et al.*, 1991a; Rapiejko and Gilmore, 1992). Another subunit, the SRP19 homolog, has been cloned from *S. cerevisiae* and characterized (Reid, 1991; Stirling and Hewitt, 1992; Stirling *et al.*, 1992). It is encoded by the *SEC65* gene and represents a 31.2 kDa protein which is responsible for the stable association of the SRP54p with the SRP (Hann *et al.*, 1992). The yeast SRP represents a 16S particle that apart from its protein constituents contains a small cytoplasmic RNA scR1 (Hann and Walter, 1991). Both protein subunits of the yeast SRP identified so far are not absolutely necessary for cell growth in certain *S. cerevisiae* strains, although cells with deletions in the corresponding genes grow poorly (C.S. Stirling, personal communication).

The affinity of the signal recognition particle for the signal peptide depends on the nascent chain length and drops with increasing length (Siegel and Walter, 1988). There is evidence that not all proteins that enter the ER are imported via SRP mediated translocation, which is corroborated by the subsequent finding that there are two translocation pathways (Greene *et al.*, 1992; Rapoport, 1990; Rapoport, 1991). The hypothesized role of the SRP in translational arrest of nascent secretory proteins has

been suggested to be an artefactual observation in wheat germ cell-free systems (Meyer, 1985), although subsequent investigations could show that translational arrest was not unique to wheat germ translation extracts and was mediated by the SRP (Wolin and Walter, 1989). *In vivo* evidence for translational arrest has been provided by analysis of a mutation in the 7S RNA of the SRP of *Yarrowia lipolytica* which arrests translation (Yaver *et al.*, 1992). Translocation in yeast of some proteins (e.g. α-mating factor) can occur post-translationally and not involving SRP. It is not known whether this process is mediated by polypeptide chain binding proteins (Rothman, 1990).

Ribosome binding to the ER is dependent on a protease-sensitive factor and is believed to be mediated by a ribosome acceptor which so far has escaped successful purification (Collins and Gilmore, 1991; Meyer, 1991; Nunnari *et al.*, 1991; Rapoport, 1991). The SRP receptor located in the ER membrane consits of two subunits. The αSRP receptor subunit has an essential GTP binding site participating in efficient translocation (Rapiejko and Gilmore, 1992; Wiedmann *et al.*, 1987). This subunit has also been hypothesized to represent part of the protein conducting channel based on its electric properties (Simon and Blobel, 1991). The assembly of the α and β subunits of the SRP receptor on the ER membrane in mammalian cells are independent of SRP mediated targeting. It occurs in a two-step mechanism involving the targeting and subsequent anchoring of the α subunit to the ER membrane (Andrews *et al.*, 1989). Ribophorins I and II are believed to be in close proximity to the site of translocation but not part of the translocation channel (Rapoport, 1991; Yu *et al.*, 1990).

In vitro translocation assays demostrated that ATP, membrane proteins and cytosolic proteins are necessary for translocation into the ER (Hansen *et al.*, 1986; Sanz and Meyer, 1989; Waters and Blobel, 1986; Waters *et al.*, 1986). ATP is necessary for assembly but not required for the translocation process itself (Lévy *et al.*, 1991). Four translocation deficient mutants have been isolated (Deshaies and Schekman, 1987; Deshaies and Schekman, 1989; Deshaies *et al.*, 1989; Rothblatt *et al.*, 1989; Stirling *et al.*, 1992; Toyn *et al.*, 1988). Further analysis established that they are associated in a complex, also with two other protein species (Deshaies and Schekman, 1990; Deshaies *et al.*, 1991). In yeast, BiP function is required for complete translocation of proteins

into the ER (Nguyen *et al.*, 1991). Sec61p, Sec62p and Sec63p are associated with the ER membrane (Stirling *et al.*, 1992; Deshaies and Schekman, 1990; Sanders *et al.*, 1992). Coprecipitation data suggest that Sec62p and Sec63p exist together in a complex with a 31.5 kDa glycoprotein and a 23 kDa protein (Deshaies *et al.*, 1991). Biochemical coss linking revealed that Sec61p also exists in a complex with the proteins. In addition the heat shock proteins Ssa1p, Ssa2p and BiP participate in translocation. A recent study addressing the molecular basis of the translocation process in yeast dissected several sequential processes before and during translocation of polypeptides (Müsch *et al.*, 1991; Saunders *et al.*, 1992). The precursor protein makes contact via its signal peptide to Sec61p in an ATP dependent manner. Contact with Sec62p, Sec63p and BiP occurs simultaneously or just prior to that process. BiP functions a second time to advance the secretory protein from an immature complex with Sec61p to a mature complex, resulting in translocation across the ER membrane. These findings corroborate previous observations that nascent chain translocation, targeting and signal sequence insertion are independent processes (Nicchitta and Blobel, 1989). Not all proteins that are translocated into the ER depend on Sec61p and Sec63p (Green *et al.*, 1992). Initial mutations in *SEC61*, *SEC62* and *SEC63* are not impaired in the assembly and glycosylation of type II membrane proteins (Stirling *et al.*, 1992). A different approach screening for import defective integral membrane proteins also identified *SEC61* and and a new gene, *SEC65* (see above).

Studies in mammalian cells by cross-linking of secretory proteins in transit revealed that the 34 kDa signal sequence receptor (SSRα) identified previously (Hartmann *et al.*, 1989; Ichimura *et al.*, 1992; Prehn *et al.*, 1990) is not involved in signal sequence recognition but defines the translocation site together with the 22 kDa SSRβ (Görlich *et al.*, 1990; Rapoport, 1991). Cross linking of nascent signal peptides with controlled reconstituted microsomal membranes identified a 36 kDa protein termed TRAM (for translocating chain-associating membrane protein) in close contact with the translocating signal peptide. Whether this protein is identical to the previously identified 37 kDa protein associated with type I signal anchor domains (High *et al.*,

1991b) or the 39 kDa protein encoding the putative translocation tunnel (Krieg *et al.*, 1989; Thrift *et al.*, 1991) is not known.

Removal of the signal sequence occurs upon or immediately after translocation and is catalyzed by a signal peptidase located in the ER lumen. The yeast signal peptidase is a multimeric complex consisting of the *SEC11* gene product and at least one glycoprotein with a molecular mass of 25 kDa (Böhni *et al.*, 1988; YaDeau *et al.*, 1991).

Proteins entering the ER exhibiting the Asn-X-Thr/Ser (X all amino acids but Pro) N-linked glycosylation consensus motif are modified by the addition of a complex oligosaccharide to the amino group of asparagine (Abeijon and Hirschberg, 1992; Kornfeld and Kornfeld, 1985). Other important glycosylation reactions also are located in the ER, such as the addition of glucose residues to high-mannose glycoproteins (Hirschberg and Snider, 1987; Kukuruzinska *et al.*, 1987; Trombetta *et al.*, 1991), the addition of N-acetylglucosamine residues to Ser or Thr (O-linked glycosylation) (Abeijon and Hirschberg, 1988; Strahl-Bolsinger and Tanner, 1991), and lastly the incorporation of glucosamine and mannose into the glycosylphosphatidylinositol (GPI) anchor of membrane associated proteins (Conzelmann *et al.*, 1990; Conzelmann *et al.*, 1992; Doering *et al.*, 1990). The dolichol-linked oligosaccharide precursor is synthesized on the cytoplasmic orientated ER membrane (Abeijon and Hirschberg, 1992). The stepwise addition of GlcNAc and Man to the dolichol-lipid anchor occurs until $GlcNAc_2Man_5$ is formed. The oligosaccharide is attached to the dolichol via a diphosphate linkage. At this stage translocation into the ER lumen occurs. The $GlcNAc_2Man_9Glc_3$ core-oligosaccharide is synthesized in the ER lumen by addidion of four more mannose residues and subsequent addition of three glucose residues. The dolichol-P-P-$GlcNAc_2Man_9Glc_3$ acts as donor of the oligosaccharide to the nascent protein, after which the remaining dolichol-P returns to the cytoplasmic side of the ER membrane. Three yeast ER glycosyl transferases as well as the Sec59 protein have a 13 amino acid membrane spanning peptide in common that specifically recognizes the isoprenoid region of dolichol phosphate (Albright *et al.*, 1989). The fact that mutations in *SEC53* and *SEC59* are conditionally lethal suggests that some aspect of glycosylation

is an essential process (Bernstein *et al.*, 1989b; Feldman *et al.*, 1987). Sec59p is located in the ER membrane and is involved in the folding and glycosylation of proteins in the ER lumen. The core oligosaccharide is modified by glucosidases I and II which trim the mannose linked glucose, and subsequently by an α-mannosidase which trims 1 mannose residue (Roth *et al.*, 1990). The removal of the glucose residues from the core oligosaccharide is an essential reaction in mammalian cells, but is not essential in yeast (Esmon *et al.*, 1984).

In mammals, O-linked glycoproteins receive N-acetylglucosamine from N-acetylglucosamine transferases on the cytoplasmic as well as the lumenal side of the ER membrane using UDPGlcNac as a donor. In yeast the first mannose is transferred from dolichol-P-Man to serine or threonine residues, and up to 4 additional mannose residues are transferred from GDP-Man in the Golgi (Kukuruzinska *et al.*, 1987). A number of cell surface proteins are attached to the membrane via a GPI anchor. This consists of phosphatidylinositol-glucosamine-mannose$_3$-ethanolamine phosphate of which the ethanolamine component is linked to the α-carboxyl group of the C-terminal amino acid of the protein. The mannose constituents of the GPI anchor are synthesized and attached involving phosphomannomutase, an essential yeast protein (Sec53p) (Conzelmann *et al.*, 1990). The phosphatidylinositol lipid moiety of the anchor is exchanged with a ceramide in a post ER compartment during protein traffic (Conzelmann *et al.*, 1992).

The fate of most proteins entering the ER is secretion. Resident ER proteins can exit the ER but are retrieved from a subsequent compartment, probably identical with the *cis* Golgi compartment (Hsu *et al.*, 1991; Pelham, 1988; Pelham, 1989a). Retention of resident ER enzymes is cell type dependent, and certain cell lines have normally ER located enzymes present in a post Golgi compartment (Brada *et al.*, 1990). In *S. cerevisiae*, the C-terminal tetrapeptide HDEL functions as an ER retention signal that is necessary and sufficient for localization of proteins in the ER (Pelham *et al.*, 1988; Pelham, 1990; Warren, 1990a). HDEL carrying proteins are able to exit the ER and are retrieved from an early Golgi department judged by their Golgi specific carbohydrate modification by α1-6 mannosyl transferase but not by α1-3 mannosyl transferase (Dean and Pelham, 1990). Since retention is a saturable event, it is likely to be mediated by a

specific sorting receptor. Two mutants defective in ER retention, *erd1* and *erd2*, have been isolated (Pelham *et al.*, 1988). Erd1p acts in the Golgi compartment and is required for correct glycoprocessing in the Golgi apparatus. The Golgi defect probably accounts for its *erd* phenotype and provides indirect evidence that the retrieval of ER proteins occurs in the Golgi apparatus. The protein product encoded by *ERD2* represents the sorting receptor for luminal ER proteins. The binding specificity of the sorting apparatus has been altered by introducing point mutations into the putative binding site of Erd2p (Semenza and Pelham, 1992). Expression of the *Kluyveromyces lactis ERD2* gene in *S. cerevisiae* allows the retention of proteins with C-terminal DDEL, usually not recognized by the *S. cerevisae ERD2* encoded receptor (Lewis *et al.*, 1990; Semenza *et al.*, 1990). A human homolog, hERD2, with similar properties has been identified (Lewis and Pelham, 1992) which differs from a 72 kDa protein also believed to participate in retention (Vaux *et al.*, 1990). Mutations in the hERD2 receptor alter its ligand specificity providing further evidence that hERD2 protein is the KDEL receptor and not a protein disturbing the sorting process. C-terminal KDEL sequences do not absolutely retain proteins in the ER but retard their secretion several-fold (Zagouras and Rose, 1989).

Membrane associated proteins usually resident in the ER have different signals present in the form of complex spatial arrangements and charged residues adjacent to transmembrane spanning domains, including a conserved Lys in the -3 position and a positively charged amino acid in the -4 or -5 position from the C-terminus (Gabathuler and Kvist, 1990; Jackson *et al.*, 1990). Unlike the retention of soluble ER proteins, the retention of transmembrane ER proteins is not saturable, arguing against a receptor mediated process (Jackson *et al.*, 1990). So far only one transmembrane ER protein, Sec20p, has been analyzed in yeast which has the C-terminal consensus HDEL and is sorted by the HDEL retrieval system (Sweet and Pelham, 1992). It has been suggested that the protein acts in ER to Golgi transport and subsequently is retrieved from the Golgi to function in a subsequent round of vesicle traffic. Why is Sec20p retrieved from the Golgi apparatus rather than retained like other transmembrane proteins resident in the ER? One possible explanation is that Sec20p associates with the cytoskeleton to

mediate efficient transport to the Golgi and then is retrieved by the HDEL receptor (Sweet and Pelham, 1992). Annother possibility is that Sec20p functions in the retrieval or budding of retrograde transport vesicles from the Golgi. This may occur by associating with filaments because a genetic interaction between *sac1* and *sec20* has been shown (Cleves *et al.*, 1989). This hypothesis would explain the *erd* pheneotype observed in *sec20* mutants at the non-restrictive temperature.

The ER is involved in degradation of aberrant proteins in an orderly, Ca^{2+} dependent fashion (Tsao *et al.*, 1992). The selective breakdown of newly synthesized proteins retained within the ER is mediated by the specific recognition of structural features by components of the degradative system (Bonifacino *et al.*, 1991; Stafford and Bonifacino, 1991). The introduction of two basic amino acids in certain positions into the transmembrane domain of the α chain of the multi-subunit T cell antigen receptor will result in retention and rapid degradation of the protein in the ER. These and similar studies (Cosson *et al.*, 1991) have shown that membrane protein association can occur by adequately located opposite intramembrane charge pairs. Some cell lines have specific resident ER proteins that bind to protein monomers and protect them from proteolytic cleavage before they form homopolymers (Clarke *et al.*, 1991) or that the formation of heteropolymers prevents the degradation of labile proteins (Wileman *et al.*, 1990a; Wileman *et al.*, 1990b; Wileman *et al.*, 1991a; Wileman *et al.*, 1991b). The correct folding of proteins seems to be crucial for subsequent transport from the ER (Rose and Doms, 1988). Misfolded proteins are bound to binding protein (BiP) and are selectively retained in the ER until proteins adopt the correct folded conformation (de Silva *et al.*, 1990; Dorner *et al.*, 1987; Hurtley and Helenius, 1989; Marquardt and Helenius, 1992; Navarro *et al.*, 1991; Singh *et al.*, 1990; Suh *et al.*, 1989; Taniyama *et al.*, 1992). BiP can exist in three differnt modified forms depending on the metabolic state of the cell, complexed to other proteins, as free unmodified monomer or as free phosphorylated aggregates (Freiden *et al.*, 1992). This indicates a putative modulation other than by induction of biosynthesis in the presence of incorrectly folded proteins in the ER. There is evidence that Ca^{2+} acts in the retention of reticuloplasmins in mammalian cells, and the introduction of Ca^{2+} ionophores will result in the secretion of

reticuloplasmins rather their selective retention in the ER (Booth and Koch, 1989). Ca^{2+} also can change the oligomerization properties of ER proteins (Poruchynsky *et al.*, 1991; Sambrook, 1990). A decrease in Ca^{2+} levels can also dissociate proteins bound to BiP and thus no longer prevent the retention of incorrectly folded proteins (Suzuki *et al.*, 1991). Depletion of Ca^{2+} can also accelerate protein degradation in the ER (Wileman *et al.*, 1991a; Wielean *et al.*, 1990c).

BiP mediated retention of misfolded or incompletely assembled proteins involves the exit from the ER and subsequent retrieval from the cis Golgi compartment (Hsu *et al.*, 1991). The problem of how BiP can distinguish folded from unfolded proteins has been directly addressed by identifying peptide sequences that BiP binds to (Flynn *et al.*, 1991). Heptameric random polypeptides bound preferentially but not exclusively to BiP when alipathic amino acids were present. A comparison of energy fingerprints between the heptamers and proteins suggests that BiP will bind to stretches of amino acids usually found inside folded proteins but also with smaller affinity to other random peptide sequences. The ATP requirement for activation of BiP is matched by translocation of ATP into the ER lumen via a specific translocator (Clairmont *et al.*, 1992).

1.6.2. ER to Golgi Transport

Transport from ER to Golgi is mediated by vesicular intermediate transport vesicles (Beckers *et al.*, 1990; Groesch *et al.*, 1990; Hicke and Schekman, 1990; Holcomb *et al.*, 1987; Kaiser and Schekman, 1990; Lodish *et al.*, 1987; Rexach and Schekman, 1991; Warren, 1990b; Wilson *et al.*, 1991) and can be divided into at least three distinct steps: vesicle budding, vesicle targeting and vesicle fusion which are all defined by distinct gene products and requirements in an *in vitro* assay. Epistasis analysis identified mutants which are defective in budding off vesicles from the ER (*sec12*, *sec13*, *sec16* and *sec23*) and fusion of vesicles with the Golgi (*sec17*, *sec18* and *sec22*) (Kaiser and Schekman, 1990). Several mutations are lethal in combination, suggesting cooperation between different gene products. ER derived vesicles (EDVs)

bud off from the endoplasmic reticulum in a defined manner involving several gene products (d'Enfert *et al.*, 1991a; Melançon *et al.*, 1991; Rexach an Schekman, 1991). The function of Sec12p and Sar1p has been closer examined. Sec12p is a type II integral membrane protein resident in the ER which genetically and physically interacts wih Sar1p (d'Enfert *et al.*, 1991b; Nakano *et al.*, 1988). Sar1p attaches to the membrane via Sec12p and mediates vesicle budding during or immediately before GTP hydrolysis (d'Enfert *et al.*, 1991b). Detailed insight about the secretory pathway comes from *in vitro* reconstitution assays allowing the dissection of individual steps of vesicle budding and fusion (Baker *et al.*, 1988; Baker *et al.*, 1990; Beckers *et al.*, 1990; Balch *et al.*, 1987; Haselbeck and Schekman, 1986; Oka *et al.*, 1991; Rexach and Schekman, 1991; Ruohola *et al.*, 1988; Schwaninger *et al.*, 1991). Whether an intermediate functional compartment exists between the ER and the cis Golgi compartment (Schweizer *et al.*, 1991), or whether transport vesicles represent this compartment is not known. Transport is dependent on ATP, cytosol and is inhibited by GTPγS. It also depends on functional *SEC* gene products (Baker *et al.*, 1988; Baker *et al.*, 1990; Rexach and Schekman, 1991). A number of GTPases have been found to regulate specific stages of vesicle budding, targeting and fusion in the secretory pathway by a mechanism that is different from that of signal transduction (Balch, 1990; Bourne, 1988). The transport steps from ER to Golgi are no exception, involving Arf1p, Bet1p, Bos1p, Bos2p, Sar1p and Ypt1p (Nakano and Muramatsu, 1989; Newman and Ferro-Novick, 1987; Nigam, 1990; Oka *et al.*, 1991; Shim *et al.*, 1991). Excepting Sar1p, these small GTP binding proteins (smgs) have not been studied in detail. Based on the genetic interaction between their structural genes, Bet1p, Bos1p and Sec22p probably interact to promote transport from the ER to the Golgi complex (Newman *et al.*, 1990). GTPases have a conserved structure and function associated with conformational changes during GTP hydrolysis (Downward, 1990; Jurnak *et al.*, 1990; Privé *et al.*, 1992; Uhl *et al.*, 1990; Wittinghofer and Pai, 1991). Most undergo isoprenylation dependent membrane attachment via their C-terminal CXXX tetrapeptide (Kinsella and Maltese, 1991; Spiegel *et al.*, 1991). It has been suggested that smgs participate in conferring directionality of the reaction step they mediate similar to the action of EF-Tu in protein synthesis (Bourne *et al.*, 1990;

Bourne *et al.*, 1991; Limmer *et al.*, 1992; Plutner *et al.*, 1991). This hypothesis has not been tested yet since no *in vitro* system was available to follow the proposed molecular switch of the smgs.

Ypt1p, a *ras*-like small GTP binding protein whose GTP binding domain is essential for functioning probably confers targeting information to the EDVs (Segev *et al.*, 1988; Wagner *et al.*, 1987). *ypt1* mutants are defective in transport between ER to Golgi and loss of Golgi function (Bacon *et al.*, 1989). While the loss of Ypt1p function *in vivo* is repressible by the addition of Ca^{2+}, the Ypt1p function in an *in vitro* transport reaction can only be restored by the addition of cytosol by not by Ca^{2+}. This argues for the primary function of Ypt1 protein in transport (Bacon *et al.*, 1989; Baker *et al.*, 1990) rather than the regulation of Ca^{2+} levels (Schmitt *et al.*, 1988) or the cell cycle (Segev and Botstein, 1987). The *ypt1* mutation can also be suppressed by mutations in ATPases reducing the cytoplasmic Ca^{2+} concentration (Rudolph *et al.*, 1989). Ypt1p GTPase activity is regulated by a specific effector, yptGAP, a protein interacting with the effector domain of Ypt1p (Becker *et al.*, 1991). The yptGAP has not been identified, but a homologous isolate from mammalian cells is active with Yp1p or rab1p as specific substrates. There is evidence that $p21^{ras}$ is affected by different GAP activities (Bollag and McCormick, 1991). Whether GTP hydrolysis in smgs in the secretory pathway are subject to stimulation by GAPs is not known, nor is the function of GAPs in the secretory pathway. Four suppressors of a Δ*ypt1* strain, *SLY1*, *SLY2*, *SLY12* and *SLY41*, have been isolated and characterized (Dascher *et al.*, 1991). While *SLY12* is identical to *BET1* a gene involved in vesicle transport and fusion, the nature of the other suppressor genes in not known although they might function in the regulation of Ypt1p activity and genetically interact (Ossig *et al.*, 1991). *sly2* mutants accumulate aberrant ER structures at a non-permissive temperature.

Sec7p, a protein previously reported to act within vesicular Golgi traffic, has recently been shown to be involved in ER to Golgi transport (Franzusoff *et al.*, 1992). Sec7p coats ER to Golgi intermediate transport vesicles and anti-Sec7p antisera inhibited transport in an *in vitro* transport reaction. *sec7* mutants accumulate Golgi structures at the nonpermissive temperature indicative of a Golgi transport defect which

has been confirmed by a biochemical Golgi analysis (Franzusoff and Schekman, 1989). The combined results suggest that Sec7p acts at multiple stages in vesicular transport including the step from ER to Golgi, and that there may be a bypass for ER to Golgi transport because Golgi compartments accumulate despite the transport defect from ER to Golgi. Alternatively, the anti-Sec7p antisera could recognize features of ER to Golgi intermediates by cross-reactivity or by direct association with phospholipid epitopes. For instance, Uso1p is a protein hypothesized to function in the ER to Golgi transport pathway (Nakajima *et al.*, 1991). The protein is very similar in size to Sec7p and also is a highly charged protein. Since highly charged regions in proteins are likely to be highly antigenic, antibodies binding to Sec7p could recognize other proteins with similar regions, although in case of Sec7p the antibody was raised against a region of the protein which did not include the extremely acidic N-terminal region of Sec7p (Franzusoff *et al.*, 1991a). This hypothetical cross-reactivity of the antibody between Sec7p and Uso1p could account for the immuno-isolation of EDVs.

Sec23p acts in the cytoplasm as a member of a multiprotein complex to promote protein transport from the ER to the Golgi (Hicke and Schekman, 1989; Orci *et al.*, 1991a). Other cytosolic factors in the intermediate transport have been identified (Wattenberg *et al.*, 1990). Efficient ER to Golgi traffic requires K^+ ions for fusion of EDVs with the Golgi apparatus (Judah *et al.*, 1989), as well as Ca^{2+} ions and ATP (Kuznetsov *et al.*, 1992; Rexach and Schekman, 1991). The molecular function underlying the cation requirements remains obscure. There is evidence that efficiency of transport is determined by cytoskeletal factors such as Uso1p (Nakajima *et al.*, 1991). Whether such proteins act as a motor from the ER to Golgi as basis for vesicle transport is unlikely because transport reactions can be reconstituted *in vitro* in the absence of microtubules. It appears likely that Uso1p is a coat protein of EDVs or involved in scision processes. Evidence from mammalian cells suggests that a 58 kDa Golgi membrane protein migrates along microtubules from ER to Golgi and might bind ER and Golgi vesicles to microtubules to allow efficient transport (Saraste and Svensson, 1991; Saraste *et al.*, 1987).

Fusion of EDVs with the Golgi apparatus requires the presence of the *SEC18* gene product, the yeast equivalent of the N-ethylmaleimide sensitive factor (NSF) (Beckers *et al.*, 1989; Wilson *et al.*, 1989). The same fusion protein is required for subsequent fusion events within the Golgi apparatus (Malhotra *et al.*, 1988) and endocytic vesicle fusion events (Diaz *et al.*, 1989). There is evidence that Sec18p might be required for transport of some proteins but not for others. The transport of the high glucose affinity carrier depends on functional *SEC* gene products and is not localized to the plasma membrane in a number of *sec* mutants with the exception of *sec18* cells (Bisson, 1988).

Not all vesicles derived from the ER are transported to the Golgi but some participating in cholesterol transport can bypass the Golgi apparatus and fuse with the plasma membrane directly (Urbani and Simoni, 1990).

1.6.3. Golgi Apparatus

The Golgi represents a defined structure in mammalian and plant cells with several cisternae arranged in an orderly fashion (Rambourg *et al.*, 1981; Rothman, 1981). The Golgi in yeast cannot be visualized by electron microscopy under physiological conditions in wild type cells, probably because the membrane traffic occurs at a high turnover rate. It does, however, form cisternae in *sec7* and *sec14* mutants at a non-permissive temperature in low glucose medium. Using antibodies against Sec7p and Kex2p, the Golgi apparatus has been immunolocalized in *S. cerevisiae* (Franzusoff *et al.*, 1991b). The molecular mechanism of transport through the Golgi complex is higly conserved between yeast and mammalian cells as demonstratd by the rescue of *in vitro* intercompartmental transport of cytosol deprived Golgi membranes from mammalian cells by yeast cytosol (Dunphy *et al.*, 1986). The kinetics of glycoprotein as well as glycolipid transport are very similar and thought to occur by the same mechanism (Dawidowicz, 1987; Wattenberg, 1990).

A novel 58 kDa protein has been identified in mammalian hepatocytes that is located on the Golgi membrane and associates with microtubules *in vitro* and *in vivo* (Bloom and Brashear, 1989). Although microtubules maintain structural integrity of the

Golgi compartment, they are not required in the determination of polarity or vesicular traffic (Iida and Shibata, 1991; Kreis, 1990; Skoufias *et al.*, 1990; Turner and Tartakoff, 1989). Time lapse photography revealed that tubulovesicular processes emerge from the *trans* Golgi and migrate along microtubules to link with other *trans* Golgi elements. At the same time vesicular structures bud off the Golgi and migrate along microtubules at a comparable rate (Cooper *et al.*, 1990). These tubules do not inter-connect ER, *cis* and middle Golgi, imposing the question why heterologous compartments are unable to fuse in a comparable manner.

The Golgi apparatus in eukaryotes undergoes mitosis dependent reorganization to ensure its inheritance (Warren, 1989). It vesicularizes during mitosis and the second meiotic metaphase, and processes such as secretion are arrested (Colman *et al.*, 1985). However, transport from ER to Golgi is not disabled (Ceriotti and Colman, 1989). This implies that the structural feature "Golgi" is not important at least in accepting ER derived transport intermediates and fusion still can faithfully occur in a vesicularized Golgi. Why do cells have a highly structured Golgi if certain reactions can be maintained in vesicular Golgi compartments, like in *S. cerevisiae*? One possibility is that transport between stacked compartments is more efficient and allows the stepwise progression of secretory enzymes more quickly than would be the case in randomly distributed organelles. The mechanism underlying the vesicularization of the Golgi apparatus is speculative at present. A 57 kDa Golgi associated protein has been identified that undergoes mitosis dependent phosphorylation and relocation (McMorrow *et al.*, 1990). The phosphorylation is mediated by $p34^{cdc2}$, and a model has been proposed that relies on the continuation of budding of Golgi vesicles while fusion is inhibited as a result of the phosphorylation (Warren, 1989). Fragmented rat Golgi can reassemble in *Xenopus* oocytes raising hopes that the assembly process can be reconstituted (Paiement *et al.*, 1989).

The Golgi apparatus in yeast has been examined biochemically and represents at least three distinct membrane enclosed compartments (Franzusoff and Schekman, 1989b). The *cis* and *trans* compartments are defined by α1-6 mannosyltransferase I activity and Kex2p activity (Julius *et al.*, 1983; Redding *et al.*, 1991; Wilcox and Fuller,

1991), respectively, and can be separated by density gradient fractionation (Cunningham and Wickner, 1989). A number of sequential glycosylation reactions involving a number of α1-6, α1-2 and α1-3 mannosyltransferases occur in the Golgi but whether each requires a separate membrane enclosed compartment is not known (Farquhar, 1985; Fransuzoff and Schekman, 1989; Hirschberg and Snider, 1987; Kornfeld and Kornfeld, 1985; Kukuruzinska *et al.*, 1987; Tanner and Lehle, 1987). In mammals and yeasts, core glycosylated glycoproteins are terminally modified by galatosyltransferases located in a late Golgi compartment (Chappell and Warren, 1989; Watzele *et al.*, 1991). Also, tyrosine sulfation, a modification promoting efficient transport, takes place in the *trans* Golgi compartment (Friederich *et al.*, 1988). The *trans* Golgi in yeast represents the compartment where the sorting of vacuolar proteins takes place. The maturation of the α-factor precursor involving the excision of the four repeated peptide sequences representing the mature peptide (Kurjan and Herskowitz, 1982) is catalyzed by the *KEX2* protein product (Achstetter and Wolf, 1984; Julius *et al.*, 1984), a reaction which has been localized to a late Golgi compartment. Dipeptidyl aminopeptidase A, another enzyme participating in the proteolytic processing of the α-factor, is localized in the Golgi apparatus, as is carboxypeptidase yscα (Achstetter and Wolf, 1984). Together the three proteinases process the α-factor precursor to the mature 13 amino acid secreted polypeptide. DPAP A and Kex2p are localized in the late Golgi and are retained in this compartment by a retention signal (Raymond *et al.*, 1992). The Kex2p compartment has been identified to be the sorting compartment where vacuolar proteins are sorted from secretory proteins (Graham and Emr, 1991). Recycling of resident Golgi proteins might occur analogously to the ER recycling pathway and there is evidence that Kex2p is recycled from an intermediate compartment between the trans Golgi and secretory vesicles, possibly involving clathrin coated vesicles (Bowser and Novick, 1991; Payne and Schekman, 1989). Whether the recycling occurs via the plasma membrane or through an intermediate vesicular recycling compartment is not known.

Intra Golgi traffic has been reconstituted *in vitro* (Pfeffer and Rothman, 1987; Rothman and Orci, 1992). It is mediated by non-clathrin coated vesicular transport

intermediates that bud off and fuse with the subsequent comparment and is dependent on a number of proteinous factors, GTP, ATP and Ca^{2+} (Farquhar, 1985; Kobayashi and Pagano, 1988; Malhotra *et al.*, 1989; Orci *et al.*, 1989; Persson *et al.*, 1988; Persson *et al.*, 1992; Rothman and Orci, 1992). It shares the requirement for Sec18p and Sec23p with the transport from ER to Golgi, indicative that the two protein products mediate processes common to general budding and fusion processes in different compartments (Graham and Emr, 1991). Intra Golgi vesicles have been purified and their coat proteins isolated (Malhotra *et al.*, 1989; Serafini *et al.*, 1991). The coat proteins (COPs) include α-COP (170 kDa), β-COP (110 kDa), γ-COP (98 kDa), δ-COP (61 kDa) and the smg ARF1p (21 kDa). Purification of native β-COP showed that it existed in an approximately 700 kDa complex, the coatomer, which promotes vesicle budding (Rothman and Orci, 1992). The protein also shares homology with β-adaptin (Duden *et al.*, 1991b). Coat proteins are found in yeast, including α-COP (Sec16p), β-COP (p105 bound to Sec23p), γ-COP (Sec23p), δ-COP (Sec21p), p36 (Sec13p), p20 (Sec12p) and ARF1, arguing for a conserved mechanism of non-clathrin coated vesicle formation (Rothman and Orci, 1992). Clathrin coated vesicles associated with the Golgi apparatus link the clathrin coat to membrane associated proteins via a γ-adaptin/β'-adaptin dimer. Based on protein sequence analysis the γ-adaptin is similar to α-adaptin mediating binding of clathrin to coated pits (Robinson, 1990) and probably performs a similar function.

Selective depletion with controlled reconstitution of Golgi membranes has identified three peripheral membrane proteins required for vesicular transport including NSF and two fractions of 500 kDa (Fr1) and 40 kDa (Fr2) (Clary and Rothman, 1990). The fusogenic activity has been attributed to three individual closely related proteins of 35, 36 and 39 kDa, α-SNAP, β-SNAP and γ-SNAP, respectively (SNAP for soluble NSF attachment proteins) which is capable of binding to Golgi membrane as well as an integral membrane receptor (Clary *et al.*, 1990; Weidman *et al.*, 1989). NSF only binds to SNAPs in the presence of the integral receptor to form a 20S multisubunit protein complex (Wilson *et al.*, 1992). γ-SNAP has a distinct binding site to that of α- and β-SNAP, which themselves are equivalent, alternative subunits of the particle. The correct

assembly of this protein complex preceeds the actual fusion process. Also, fatty acylation promotes the budding and fusion of transport vesicles with Golgi cisternae by an unknown mechanism (Pfanner *et al.*, 1989; Pfanner *et al.*, 1990). The fatty acyl-CoA is required after uncoating and NSF binding but before the actual fusion process. The same pathway operates in yeast based on the finding that Sec17p and α-SNAP are exchangeable. In Golgi membranes α- and β-SNAP bind competetively to a common site, while γ-SNAP binds to a different site (Rothman and Orci, 1992). The α/β-SNAP membrane protein receptor is either the same protein as the γ-SNAP receptor or is non-covalently associated with it. Upon Golgi membrane association the SNAP complex can associate with NSF to form a fusogenic complex which disassociates after ATP hydrolysis, possibly of the NSF bound ATP. The molecular mechanism of membrane fusion is not understood, and it appears that a number of proteins have to lower the repulsive forces between different membrane compartments (Stegmann *et al.*, 1989).

smgs also perform an essential role in *S. cerevisiae* and mammalian inter Golgi transport (Goud *et al.*, 1990). rab6p and ARF1p are two mammalian smgs hypothesized to act within the Golgi stack to promote some unknown function (Rothman and Orci, 1992). It can be envisaged that they act analogously to Sar1p in ER to Golgi vesicle budding. ARF1p has been suggested to cycle between membrane bound (myristoylated) and cytosolic forms dependent on the uptake or hydrolysis of GTP. It might associate and disassociate coat proteins during this cycle. All rab homologues are hypothesized to bear targeting information. Trimeric G-proteins are also involved in the formation of secretory vesicles from the trans Golgi network as has been examplified by $[AlF4]^-$ inhibitor studies (Barr *et al.*, 1991; Kahn, 1991) and exert inhibitory control on transport vesicle coating and vesicle formation.

The fact that Sec14p (Bankaitis *et al.*, 1989), a protein blocking intra Golgi transport, is identical to a phospholipid transfer protein (Bankaitis *et al.*, 1990) suggests that either Sec14p involves the retrieval of acidic phospholipids and relocalization to the ER, or that the protein delivers phosphatidyinositol (PI) and/or phosphatidylcholine (PC) to the late Golgi. Mutations in specific aspects of phosphatidyl choline biosynthesis can bypass the essential requirement for Sec14p (Cleves *et al.*, 1989;

Cleves *et al.*, 1991) suggesting that a reduction of PC in the Golgi is required to permit secretion to occur (Cleves *et al.*, 1991). The whole story is even more complicated by the fact that a genetic repressor of a *ts* actin mutation, a *sac1* mutation, also suppresses the cell's requirement for functional Sec14p. Sac1p is an actin bundling protein organizing the cytoskeleton and acts as an antagonist to Sec14p function and actin assembly. It is an integral membrane protein (Cleves *et al.*, 1989) of the ER and Golgi (A.E. Cleves, unpublished results). The mammalian actin binding proteins profilin and gelsolin have a higher affinity for PI 4-phosphate and PI 4,5-bisphosphate than actin (Goldschmidt-Clermont *et al.*, 1990). It has been proposed that such actin binding proteins sequester phosphoinositides, and that this interaction of actin binding proteins with phospholipids represents an important aspect of a mechanism by which actin polymerization and phospholipid mediated signalling may be regulated.

Studies addressing the selective retention of Golgi proteins suggest that C-terminal cytoplasmic polypeptides (Armstrong and Patel, 1991; Hopkins, 1992) as well as transmembrane domains (Armstrong *et al.*, 1990; Machamer and Rose, 1987; Machamer *et al.*, 1990; Swift and Machamer, 1991; Wong *et al.*, 1992) are responsible for the correct retention in the Golgi compartment. The first 44 amino acids of α-2,6-sialyltransferase, a type II resident Golgi protein, specifies retention in the trans Golgi and TGN (Munro, 1991). Both the transmembrane domain as well as adjacent cytoplasmic regions are involved in the selective retention. Whether these retention patches are recognized by a protein mediating retention or function in the formation of homomultimers is not known at present. The release of dimers/multimers during mitosis could be mediated by phosphorylation of the cytoplasmically orientated accessory domains which subsequently dissociate because of repulsive equal charges (G. Warren, op. citation). The cytoplasmic transmembrane spanning domains and 10 amino acids of the lumenal domain of β-1,4-galactosyltransferase are sufficient to retain a protein fusion in the Golgi apparatus (Nilsson *et al.*, 1991). The cytoplasmic domain plays an accessory role in the retention of the Golgi protein but not in determining compartmental specificity of retention. A 58 kDa transmembrane protein located in the *cis* Golgi of hepatocytes is not terminally glycosylated by the *trans* Golgi glycosyl

transferases. This provides evidence that it is not recycled from a *trans* Golgi compartment but never exits the *cis* Golgi compartment (Hendricks *et al.*, 1991).

Brefeldin A (BFA), a fungal metabolite, disrupts the Golgi stack by an unknown mechanism (Lippincott-Schwartz *et al.*, 1990) involving the selective dissociation of a 110 kDa pripheral membrane protein identical to β-COP and a so far unknown 200 kDa protein from the Golgi apparatus (Burgess *et al.*, 1991; Donaldson *et al.*, 1991; Klausner *et al.*, 1992; Lippincott-Schwartz *et al.*, 1989; Narula *et al.*, 1992; Orci *et al.*, 1991b) during which resident Golgi proteins are relocalized to the ER in a reversible reaction and budding from the ER is inhibited (Ulmer and Palade, 1989; Ulmer and Palade, 1991). Proteins retained in the ER were not sialic acid modified, although they acquired the conversion from high mannose forms to complex type structures characteristic for early and middle Golgi modifications (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). These findings together with the fact that sialyltransferase is not relocated to the ER suggest that enzymes in the *trans* Golgi and/or *trans* Golgi network are not affected by brefeldin A in the same way the *cis* and *medial* Golgi are (Chege and Pfeffer, 1990). BFA exerts a similar membrane rearrangement within the endosomal/lysosomal transport system involving the selective fusion of the central vacuolar system via tubule formation of the TGN and subsequent fusion with endosomes (Lippincott-Schwartz *et al.*, 1991; Reaves and Banting, 1992; Wood *et al.*, 1991). The association/dissociation process of the 110 kDa β-COP with the Golgi apparatus is regulated by AlF_4^- sensitive GTPases and perturbed by BFA by interference of the association process (Donaldson *et al.*, 1991). ATP also participates in the association of the 110 kDa protein with the Golgi, and a depletion led to the release of the 110 kDa protein without fusion of the Golgi with the ER (Donaldson *et al.*, 1990). GTPγS treatment could reverse ATP depletion induced disassociation of the 110 kDa protein (Donaldson *et al.*, 1991) and antagonize BFA induced retrograde transport (Tan *et al.*, 1992). Mastoparan, an activator of heterotrimeric GTPases, promotes the binding of β-COP to Golgi membranes and antagonizes the effect of BFA (Ktistakis *et al.*, 1992). Together with the fact that AlF_4^- promotes the binding of β-COP to the membrane these data suggest that the membrane association of β-COP is

regulated by heterotrimeric GTPases, possibly ARF1 (Klausner *et al.*, 1992). The failure to form non-clathrin coated vesicles might be accompanied by NSF mediated fusion of Golgi compartments with each other to allow the formation of a network (Orci *et al.*, 1991). The fusion of the BFA induced Golgi network with the ER might rely on the formation of retrograde tubules. Whether these tubules represent exaggerated retrograde tubules present in all cells (Klausner *et al.*, 1992; Lippincott-Schwartz *et al.*, 1990) or aberrant structures resulting from the missing association of the Golgi coatomer (Pelham, 1991) is not clear. It seems plausible that the coatomer in untreated cells will prevent the fusion of bulk membranes with compatible membranes and also mediate controlled vesicular budding. In BFA treated cells the coatomer is not associated with the Golgi compartments any more, and normal budding is no longer possible. The now tubular extention where vesicle budding previously occurred will fuse with a compatibele membrane in a reaction that requires ATP. This model awaits stepwise analysis *in vitro* as does identifying the target of BFA. The fusion of the central vacuolar system is not dependent on the provision of ATP (Reaves and Banting, 1992) which can be explained by the analogy that Golgi to vacuole in yeast does not require the *SEC18* encoded general fusion protein (Graham and Emr, 1991).

The validity of the above suggested pathway has been questioned because a disorganization of both cis/middle and trans Golgi after brefeldin A treatment was observed (Alcalde *et al.*, 1992). Upon removal of brefeldin A the cis/middle located proteins exit the ER from the cis/middle compartment via a vesicular intermediate. The reorganization of the trans Golgi compartment was dependent on the presence of the earlier Golgi compartments through which translocated proteins have to migrate. Since the latter studies were based on morphological observations whereas the others were based on biochemical studies this apparent discrepancy can be explained by assuming that forward transport from trans Golgi through the TGN to the cell surface still can occur thus depleting the structural feature independent of BFA action. Other evidence suggests that BFA acts selectively in some cell lines, because MDCK cells did not show the BFA dependent dissociation of the 110 kDa β-COP and subsequent morphological changes (Hunziker *et al.*, 1991). BFA has no obvious effects on protein trafficking in

the secretory pathway in yeast (R.W. Schekman, personal communication), possibly because of the limited host range of BFA or differences in the molecular target of BFA.

1.6.4. Transport from Golgi to Vacuoles/Lysosomes

The majority of *S. cerevisiae* vacuolar protein constituents described so far are delivered to the vacuole in transit through the secretory pathway (Klionsky *et al.*, 1990; Raymond *et al.*, 1992; Schekman, 1982; Stevens *et al.*, 1982). The details of sorting, delivery and function of the constituents as well as the integrated role of the vacuolar compartment will be described in more detail in Chapter 4.

The mechanism by which soluble vacuolar yeast proteins are sorted to the vacuole differs from that from mammalian cells. The recognition and receptor binding of the protein to be sorted does not depend on the recognition, binding and retrieval of proteins labelled with a mannose 6-phosphate (Kornfeld and Mellman, 1989; see Chapter 4). On the other hand only three proteins were examined for sorting and there might be other vacuolar proteins that are sorted by a mannose 6-phosphate retrieval system, since many vacuolar proteins in yeast are also phosphorylated (Raymond *et al.*, 1992). Recognition of lysosomal proteins preceeds phosphorylation of high-mannose type N-linked oligosaccharides on the 6-carbon position of specific mannose residues. This recognition is mediated by N-acetylglucoseamine-1-phosphotransferase suggesting the presence of intrinsic peptide signals in lysosomal enzymes (Kornfeld and Mellman, 1989). This domain has no conserved homology between different lysosomal proteins of the same organism and most likely represents a signal patch (Baranski *et al.*, 1990). Thus, lysosomal proteins differ from secretory proteins in the presence of a targeting domain which is recognized and modified by the phosphotransferase to generate a new signal (mannose 6-phosphate) and subsequently recognized by mannose 6-phosphate sorting receptors (Kornfeld, 1989; Kornfeld and Mellman, 1989). Vacuolar protein sorting signals of three soluble vacuolar proteins in yeast differ in primary peptide sequence, and it has been suggested that similar signal patches might form a consensus for vacuolar targeting (Klionsky *et al.*, 1990). This has recently been questioned in

experiments addressing the specificity of sorting (Paravicini *et al.*, 1992) and it appears that different vacuolar proteins are sorted by different pathways, possibly involving different sorting receptors (Stevens *et al.*, 1986). Mammalian cells express two mannose 6-phosphate receptors with different molecular mass (46 kDa and 300 kDa, also referred to as 275 and 215 kDa) which are dimerized and bind two ligand molecules per dimer (Köster *et al.*, 1991; Kornfeld and Mellman, 1989; Pfeffer, 1988; von Figura and Hasilik, 1986). The receptors are indentical in ligand binding and are co-expressed in the same cells (Bleekemolen *et al.*, 1988). The 300 kDa receptor additionally binds to the insulin-like growth factor II in a number of species (Morgan *et al.*, 1987) and also mediates endocytosis of mannose 6-phosphate containing ligands (Gartung *et al.*, 1985), a function that cell surface localized 46 kDa receptor cannot perform, possibly because it adopts a different conformation (Chao *et al.*, 1990; Hille *et al.*, 1990; Stein *et al.*, 1987). Studies involving the selective overexpression of either receptor led to the proposal that the 46 kDa receptor releases the ligand at a site from which secetion can occur while the 300 kDa receptor quantitatively retrieves the ligand and localizes it to the lysosome (Chao *et al.*, 1990). Both receptors recycle to the Golgi involving the same endosomal compartments although their distribution among these compartments is different in that the 46 kDa receptor is almost exclusively found in the Golgi complex (Matovcik *et al.*, 1990; Messner *et al.*, 1989). Both receptors are specifically redistributed upon hormonal stimuli and thus are also under endocrinal control (Damke *et al.*, 1992).

Recent evidence suggests that the mannose-6-phosphate / mannose 6-phosphate receptor mediated sorting is not the only sorting mechanism in mammalian cells (Tsuji *et al.*, 1988). Glucerobrosidase, a membrane associated lysosomal enzyme from human skin fibroblats, does not undergo oligosaccharide phosphorylation, and is still faithfully localized to the lysosome (Aerts *et al.*, 1988). Humans with I-cell disease lack mannose 6-phosphate as a result of a defective phosphotransferase. However, they only show a tissue dependent, selective secretion of a subset of lysosomal enzymes, strongly suggesting that certain proteins in certain tissues only rely on mannose 6-phosphate dependent lysosomal protein sorting (Kornfeld, 1986). Integral (see below) and

membrane associated proteins are sorted by a different mechanism not relying on phosphorylation. This seems to suggest that a number of mechanisms exist to ensure the correct localization of lysosomal proteins, some of which are tissue specific. In mammalian cells lysosomal membrane associated proteins such as acid phosphatase (LAP) are localized to lysosomes via the cell surface (Braun *et al.*, 1989), although other reports provide evidence that two other transmembrane glycoproteins are sorted in the TGN and transported directly to the lysosome thus not involving endocytosis from the plasma membrane (Green *et al.*, 1987; Matter *et al.*, 1990). The retrieval of LAP from the cell surface occurs efficiently, and is mediated by a retrieval signal located in the cytoplasmic domain of the receptor (Dintzis and Pfeffer, 1990; Peters *et al.*, 1990). Upon arrival in the lysosome, LAP is sequentially processed by two proteolytic events (Gottschalk *et al.*, 1989). The first cleavage removes most of the C-terminal cytoplasmic domain, while the second cleavage occurs in the lysosomal lumen to release LAP from the transmembrane domain. The second proteolytic event depends on the cleavage of the C-terminal cytoplasmic domain, possibly because the C-terminus controlls the homodimer formation of LAP and if not cleaved prevents accessibility of the lumenal domains. There is evidence that the introduction of a positively charged amino acid into the first membrane spanning domain of Rous Sarcoma Virus Envelope Glycoprotein will result in the lysosomal localization of the protein rather than cell surface expression (Davis and Hunter, 1987). The studies did not address whether the protein was localized to the plasma membrane and rapidly endocytosed or migrated directly to the lysosome. The 300 kDa mannose 6-phosphate receptor's C-terminal domain contains signals for rapid endocytosis and efficient lysosomal targeting (Lobel *et al.*, 1989). Lysosomal membrane associated proteins have a Tyr-containing internalization signal in their cytoplasmic domain which is part of a β-turn in solution (Bansal and Gierasch, 1991; Eberle *et al.*, 1991; Lobel *et al.*, 1989). The aromatic amino acid is presented to the cytoplasmic receptor in the structural context of a tight turn.

The formation of autophagic vacuoles is different to that of lysosomes and is induced in hepatocytes by the depletion of nitrogen (Dunn, Jr., 1990a). Autophagic

vacuoles are derived from the smooth ER and represent a smooth double membrane enclosed vesicle. Cytoplasmic contents are engulfed by the ER during autophagy and soon after generation recruit lysosomal enzymes in transition to an acidic proteolytic compartment (Dunn, Jr., 1990b; Ueno *et al.*, 1991).

1.6.5. Secretion

Secretion is the default pathway in mammalian cells, yeasts and plants (Denecke *et al.*, 1990). Secretion in *S. cerevisiae* is polarized, and closely coupled to cell surface growth and budding. It involves the polarized deposition of new cell surface material associated with a highly assymmetric disposition of the actin cytoskeleton. Factors establishing cell polarity have been identified by isolating mutants unable to bud at a restrictive growth temperature of 37°C (Adams *et al.*, 1990; Chenevert *et al.*, 1992). Their analysis showed that bud site selection are Ca^{2+} and GTP dependent processes (Chant *et al.*, 1991; Johnson and Pringle, 1990; Johnson *et al.*, 1990; Miyamoto *et al.*, 1987; Ruggieri *et al.*, 1992), and proteins participating in bud site selection and bud formation have been identified (Chant and Herskowitz, 1991; Chant *et al.*, 1991; Chenevert *et al.*, 1992). One protein, Bem1p, has been suggested to link the actin cytoskeleton to morphogenetic determinants on the cell surface (Chenevert *et al.*, 1992). A 85 kDa actin binding protein has been located to the yeast cortical actin patches at growing surfaces of the yeast cell (Drubin *et al.*, 1988). Overproduction of the 85 kDa protein results in bud formation at adjacent sites.

The actin cytoskeleton and the Ca^{2+} binding protein calmodulin colocalize with the site of bud emergence and cell growth (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Sun *et al.*, 1992). While the actin cytoskeleton is essential for cell growth, factors such as Cap2p regulating actin filament distribution are not essential (Amatruda *et al.*, 1990). The *MYO2* encoded myosin promotes vectorial vesicle transport to the bud site probably by acting as a cytoskeletal motor to transport secretory vesicles along actin cables to the site of bud development (Johnston *et al.*, 1991). This process is required for high efficiency but is not absolutely necessary for secretory

vesicle transport. Cytoplasmic dynein has been identified as a coat protein on synaptic vesicles suggesting a role for the microtubule based motor protein (Lacey and Haimo, 1992). Actin filaments have been attributed a different role in baseline secretion in intestinal goblet cells where they form a physical barrier to slow constitutive secretion (Oliver and Specian, 1990)

10 *SEC* genes (*SEC1*, *SEC2*, *SEC3*, *SEC4*, *SEC5*, *SEC6*, *SEC8*, *SEC9*, *SEC10* and *SEC15*) have been identified so far that regulate the secretory pathway from the Golgi to the plasma membrane. *SEC15* encodes a 116 kDa hydrophilic protein that associates with the plasma membrane by binding directly to a protein complex located on the cytoplasmic side (Bowser and Novick, 1991). Preliminary evidence suggests that both the *SEC8* and the *SEC10* gene products participate in attachment of Sec15p to the membrane or a protein complex. Sec15p might function in the attachment or aggregation of vesicles coated with Sec4p to the plasma membrane (Salminen and Novick, 1989). Sec4p, a small GTP binding protein, regulates secretion in *S. cerevisiae* by cycling between an active and inactive state (Salminen and Novick, 1987; Walworth *et al.*, 1989). GTPase activity of Sec4p is modulated by an GTPase-activating protein (GAP) which so far has evaded detection (Walworth *et al.*, 1992). A Sec4p functional and structural homologue, Ypt2p, has been identified in *Schizosaccharomyces pombe* (Haubruck *et al.*, 1990). The *SEC2* gene product, a 368 amino acid protein with an essential coiled coil domain and similarity to cytoskeletal proteins, probably functions in conjunction with Sec4p and Sec15p to control vesicle fusion (Nair *et al.*, 1990).

Three proteins common to different secretory vesicles in exocrine, endocrine, neural and constitutive secretion have been identified in rat (Brand *et al.*, 1991). The secretory carrier membrane proteins (SCAMPs) have an apparent molecular mass of 31, 33 and 35 kDa and are unglycosylated transmembrane proteins throughout. A ras-related GTPase has been found to function at a late step in secretion (Wilson and Wilson, 1991). Their identity to known *SEC* gene products is not known.

The rate of bulk flow from the Golgi apparatus to the plasma membrane has been determined in mammals to be 14 minutes at 30°C following the transport of truncated sphingomyelin from the cis Golgi to the plasma membrane (Karrenbauer *et al.*, 1990).

An ER lumenal marker is secreted with a half time of 18 minutes under identical conditions, allowing 4 minutes for ER to Golgi transport.

Epithelial cells have often polarized regions of the plasma membrane differing in their protein constituents. The plasma membane polarity is maintained by intracellular sorting of apical protein constituents in the trans Golgi network from the default basolateral transport pathway (Griffith and Simons, 1986; Salamero *et al.*, 1990) or the selective retrieval of transmembrane proteins destined for the apical membrane from the basolateral membrane (Simons and Wandinger-Ness, 1990). It appears that some proteins located to the basolateral membrane have positive localization information present (Hopkins, 1992). The lipid polarity observed in polar epithelial cells is maintained by sorting before reaching the membrane surface (van't Hof and van Meer, 1990). There is evidence that a subset of apical proteins recycle through the trans Golgi network in MDCK cells although its physiological function is so far subject to speculation and might aid the efficient receptor mediated sorting (Brändli and Simons, 1989; Simons and Wandinger-Ness, 1990). Also, the developmental remodeling of membrane protein distribution and subsequent sorting involves transcytosis as well as a new specificity of apical membrane protein sorting (Wollner *et al.*, 1992).

Unlike *S. cerevisiae* which only exhibits polarized constitutive secretion, neural and endocrine cells exhibit regulated and constitutive secretion, which requires a more sophisticated machinery for sorting in the TGN and regulated release of vesicle contents in secretion (Brion *et al.*, 1992; Burgess and Kelly, 1987; Koedam *et al.*, 1992; Sossin *et al.*, 1990; Sporn *et al.*, 1989). PC12 cells have two distinct regulated secretory pathways mediated by large dense secretory compartments related to chromaffin granules and by small electron-lucent vesicles (Cutler and Cramer, 1990). Acidophilic cells of the cow anterior pituitary are able to sort three secretory proteins to different secretory granules (Hashimoto *et al.*, 1987), suggesting the existence of regulated sorting and traffic pathways. In cytolytic lymphocytes the regulated secretory vesicles have characteristics of both secretory vesicles and lysosomes in terms of protein constituents emphasizing the diversity of membrane enclosed compartments in specialized cells (Burkhardt *et al.*, 1990). A related process occurs in Alzheimer brains

where lysosomal contents are released into the extracellular medium contributing to certain physiological defects (Cataldo *et al.*, 1991). Unlike in cytolytic lymphocytes, however, a general leakage of lysosomal emzymes might be primarily responsible for the deleterious defects. There is also evidence that oxysterol induced association of an oxysterol binding protein with the Golgi complex modulates transport (Ridgway *et al.*, 1992). Constitutive secretion in many cell types is the default late secretory pathway. Most vesicle intermediates in regulated secretion are coated, and their contents are sorted in the trans Golgi network (TGN) by selective aggregation (Burgess and Kelly, 1987; Tooze *et al.*, 1987). The formation of secretory vesicle subpopulations depends on ATP (Tooze and Huttner, 1990). Aggregation of specific proteins is mediated by the low pH and high Ca^{2+} environment in the TGN which permits segregation from constitutively secreted proteins (Chanat and Huttner, 1991). The release of regulated secretory vesicles depends on an external stimulus, and requires Ca^{2+}. In pancreatic acini cells the release of amylase is induced by a rab3a effector, providing evidence that the GTPases involved in membrane transport are subject to functional modulation to control transport (Padfield *et al.*, 1992). Vesicle transport in neuronal cells is subject to modulation by Ca^{2+} and the phosphorylation of rap-1b (Sahyoun *et al.*, 1991). It also depends on the function of a small GTPase, which dissociates from synaptic vesicles during exocytosis (von Mollard *et al.*, 1991).

Vesicle aggregation on the plasma membrane is thought to be mediated by annexin, cytoplasmic Ca^{2+} binding proteins and phospholipid binding proteins. Annexins I and II promote contact between vesicles via an N-terminal repeat and engage in mediating contact between phospholipid bilayers (Ernst *et al.*, 1991). The Ca^{2+} requirement could not be shown to be essential in an *in vitro* assay where exocytosis was dependent on ATP, GTP and N-ethylmaleimide sensitive factors (Miller and Moore, 1991; DeLisle and Williams, 1986; Tatham and Gomperts, 1991). There is evidence in a number of organisms that prepositioning of regulated secretory vesicles and organelles onto the plasma membrane occurs as part of a mechanism to ensure the rapid release in case of stimulation. The effector pathways regulating secretion involve different mechanisms depending on species and tissue. They include Ca^{2+}, contractile

cytoskeleal proteins, protein kinases, phosphatases and a number of cytoplasmic proteins.

Modulation of secretion can be achieved by transporting newly synthesized secretory proteins to lysosomes for degradation rather than secretion via secretory vesicles (Lenk *et al.*, 1991).

1.7. Endocytosis

The most compelling studies describing fluid phase and receptor mediated endocytosis and the endocytic organelles comes from specialized mammalian cells (Griffith, 1989). Mammalian cells internalize a variety of growth factors, nutrients and viruses through receptor mediated endocytosis (Goldstein *et al.*, 1985). Endocytosed ligands can be routed intracellularly by several ways. Epidermal growth factor (EGF) is directed to the lysosome together with its receptor, while transferrin is released from its receptor and the receptor is recycled back to the plasma membrane (Salzman and Maxfield, 1989). In polarized epithelial cells there is documented evidence for transcytosis, the transport of ligands and their receptors from the apical site of the membrane to the basolateral (Fuller and Simons, 1986; Sztul *et al.*, 1991). Studies involving fluorescent labeled transferrin and low density lipoprotein (LDL) colocalized the two ligands in the same endocytic compartment, but could demonstrate that they are differentially sorted (Dunn *et al.*, 1989). Endocytic vesicles fuse with an endosomal compartment in which LDL accumulates, but transferrin is sorted away. Recent evidence indicates that transferrin is processed intracellularly via small coated endosomal vesicles and is not delivered into large tubular endosomes, characteristic for ligand traffic to lysosomes. The small coated endosomal vesicles have sorting and recycling properties and might represent branchpoint vesicles where differential sorting of endocytosed material takes place (Eskelinen *et al.*, 1991). It has been suggested that endocytosed receptor containing vesicles preferentially fuse with the early endosome and conversely vesicles budding from tubular extensions of early endosomes preferentially fuse with the plasma membrane. Early endosomes mature during this

process and lose their competence for interaction with the plasma membrane. Instead fusion of TGN vesicles with the late endosome increases as does a recycling pathway (Stoorvogel *et al.*, 1989). The actin cytoskeleton plays a role in the rapid turnover of short lived membrane proteins in astrocytes as a result of hormonal stimulation (Farwell *et al.*, 1990). Reconstitution of the endocytic pathway following vesicular transport of the 300 kDa mannose 6-phosphate receptor from late endosomes to the TGN is dependent on GTP and soluble cytosolic proteins sensitive to N-ethylmaleimide (Gruenberg and Howell, 1989). The cytosolic factor termed ETF-1 differs from the NSF required for transport from ER to Golgi and intercompartmental transport (Goda and Pfeffer, 1991). It has been suggested that the "pre-lysosomal" compartment receives material from the TGN and early endosomes and somehow passes it on to the lysosome (Griffiths, 1989). The recycling of transferrin receptors occurs with a half-time of 15 minutes (Yamashiro *et al.*, 1984) while the recycling of endocytosed material has a half-time of 40 minutes, demonstrating the efficiency conferred by the clustering of receptors in coated pits (Koval and Pagano, 1989). Transcytosis in MDCK cells is sensitive to BFA treatment at an early step, indicative of a BFA-sensitive coat present on endocytic compartments (Hunziker *et al.*, 1991). Endocytosis occurs from both apical and basolateral surfaces of MDCK cells, and endocytosed material from both membrane domains are targeted to a common endosomal compartment (Parton *et al.*, 1989). Endosomes also may participate in the uptake of membrane impermeable components directly from the cytoplasm (Steinberg *et al.*, 1988).

Comparatative studies on the endosomal system of *Paramecium* provided evidence for a highly conserved endocytic pathway, including the coating of the phago-/pinocytotic vesicles, their uncoating and subsequent fusion with early endosomes (Allen *et al.*, 1992). The sorting of endocytosed material from plasma membrane components has been located to an endosomal compartment having striking similarity to the same process in mammalian systems. Receptor mediated endocytosis in *S. cerevisiae* does occur and plays an important role in the selective uptake of mating pheromones (Singer and Riezman, 1990; see Chapter 4). Like the analogous process in mammalian cells it is temperature dependent and requires ATP. Whether endocytosis in

yeast is coupled to the targeting of membrane associated enzymes by retrieval from the plasma membrane is not known.

While clathrin represents the major coatomer for endocytotic vesicles in species as diverse as mammals, *Paramecium* and plants (Coleman *et al.*, 1988; Gruenberg and Howell, 1989; Robinson and Depta, 1988), the clathrin homologues in yeast are dispensable for that function (Payne *et al.*, 1988; Silveira *et al.*, 1990). Studies on mammalian coated vesicle formation suggests that receptor bound ligands are spontaneously invaginated into a coated pit and the coated vesicles are detached in a reaction involving ATP, Ca^{2+} and cytosolic factors (Lin *et al.*, 1991; Moore and Anderson, 1989; Schmid and Smythe, 1991). Not all endocytosis is mediated by clathrin coated pits. Enzymes involved in the uncoating of clathrin coated vesicles present in brain extracts as well as in yeast have been identified (Gao *et al.*, 1991). Preliminary evidence suggests that clathrin directly mediates vesicle fusion in mammalian cells (Maezawa and Yoshimura, 1991).

The membrane protein composition of different endosomal compartments varies which is consistent with the view that membrane protein recycling takes place between different compartments (Beaumelle *et al.*, 1990). The endosome and lysosome differ quite drastically in protein composition suggesting the unrelatedness of the two compartments. While the processing pathways within the endosomal system have been described for different systems, one trend observable is that not all pathways are common to all cell lines. Endocytosis might very well serve a specialized function in different cells and might utilize a slightly different mechanism adapted to the function. There is also still a debate on whether endocytic transport is vesicularized similar to ER to Golgi transport or takes place by compartmental progression (Gruenberg and Howell, 1989).

1.8. Osmohomeostasis in *S. cerevisiae*

Only little information on the osmohomeostasis in *S. cerevisiae* is available, and although the vacuole has been suggested to participate in osmoregulation (Banta *et al.*,

1988) no further details of the osmoregulatory mechanism are known. A number of studies concentrated on the elucidation of the glycerol mediated osmoadaptive pathway (André *et al.*, 1991; Blomberg and Adler, 1989; Larsson *et al.*, 1990; Reed *et al.*, 1987; Singh and Norton, 1991). While most exponentially growing cells are sensitive to a reduced water potential in the form of a 1.4 M NaCl supplement to the medium, cells that enter the stationary phase are generally more resistant to the osmotic shock and remain viable, as are cells that were conditioned with 0.7 M NaCl before plating out onto YPD agar supplemented with 1.4 M NaCl. The conditioning is dependent on *de novo* protein biosynthesis and can be prevented in the presence of cycloheximide (Blomberg and Adler, 1989). The process of conditioning is referred to as acquired osmotolerance and is accompanied by the acumulation of glycerol. Glycerol and glycerol 3-phosphate dehydrogenase, an enzyme in glycerol production, show an osmotically inducible increase irrespective of the carbon source used for cell growth (André *et al.*, 1991). Although the enzyme levels are lower and subject to glucose repression if cells are grown in glucose medium, they are elevated compared to cells grown in the absence of an osmotically active component. Removal of glycerol by repeated washing with water will reduce the osmotolerant fraction by 50% as determined by survival on YPD supplemented with 1.4 M NaCl (Blomberg and Adler, 1989). Other eukaryotes, such as *Neurospora crassa*, accumulate glycerol and mannitol as a response to low water pressure (Ellis *et al.*, 1991; van Zyl *et al.*, 1990). Glycerol and arabinitol accumulate in *Debaryomyces hansenii*, a salt-tolerant yeast, to maintain a positive turgor balance at high salinity (Larsson *et al.*, 1990). A physiological overlap between osmotolerance and thermotolerance has been observed in *S. cerevisiae* (Trollmo *et al.*, 1988). Salt-sensitive mutants isolated from *Zygosaccharomyces rouxii* were defective in glycerol catabolism and accumulation (Yagi and Tada, 1988). Any vacuolar defects in these mutants were not reported.

1.9. Aim of Thesis

The study of certain aspects of vacuolar biogenesis in yeast, namely vacuolar morphogenesis and the so far unknown role of the vacuole in cellular osmohomeostasis, were subject of this thesis. Based on the finding that some vacuolar protein sorting mutants lacked a structurally intact vacuole and were also osmosensitive (Banta *et al.*, 1988), a scheme was devised to isolate osmosensitive mutants which also have vacuolar defects. After initial characterization of the mutations conferring an osmosensitive vacuolar phenotype, it was intended to clone and sequence the genes corresponding to the mutations to be able to analyse certain aspects of vacuolar biogenesis on the molecular level.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents and Supplies

All chemicals and reagents used in this study were obtained from Sigma Chemicals plc, Poole, Dorset, UK, or BDH Chemicals Ltd, Poole, Dorset, UK, unless stated otherwise. Restriction enzymes and T4 ligase were from NBL, Cramlington, Northumberland, UK. Caesium chloride, calf intestinal alkaline phosphatase and Klenow enzyme were from Boehringer Mannheim Diagnostics and Biochemicals (England) Ltd, Lewes, East Sussex, UK. Sequenase Version 2.0 sequencing kits and mung bean deletion kits were from Stratagene, La Jolla, CA 92037, USA. Agarose and LMP agarose were from Bethesda Research Laboratories Inc, Gaithersburg, MD 20877, USA. Zymolyase 100-T was from Seikagaku Kogyo Co, Ltd, Tokyo, Japan. Peptone, nutrient broth, and yeast nitrogen base without amino acids were from Difco Laboratories, Detroit, MI 48232-7058, USA, and agar and yeast extract were from Oxoid Ltd, Basingstoke, UK. Tran[^{35}S]-label was from ICN Biomedicals Ltd, High Wycombe, Buckinghamshire, UK; α-^{35}S-ATP and silica fines were from Amersham International plc, Amersham, Buckinghamshire, UK. Filterpaper was purchased from Whatman International Ltd, Maidstone, Kent, UK. Hybridization membranes were from Schleicher and Schuell, Dassel, FRG. Polaroid 667 film was from Polaroid (UK) Ltd, St. Albans, Hertfordshire, UK. Unless stated otherwise, photographic materials were from Kodak Ltd, Liverpool, UK.

Antisera to CPY, Pho8p, and the 60 kDa subunit of the vacular membrane ATPase were a gift from Tom Stevens, Oregon, USA, and antisera to PrA and PrB were a gift from Dieter Wolf, Stuttgart, FRG.

2.1.2. Phage Strains

Bacteriophage R408 (Stratagene, La Jolla, CA 92037; Russel, M. *et al.*, 1986) was used in this study as helper phage for recovering single stranded DNA from the Stratagene Bluescript™ vectors pKS and pSK for sequencing.

2.1.3. Bacterial Strains

The bacterial strains used in this study were all *Escherichia coli* and are listed in Table 2.1. All strains represented laboratory stocks, or for XL1-Blue, were purchased from commercial sources.

Strain	Genotype	Reference
HB101	*supE*44 *hsdS*20 (r_B^- m_B^-) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	Bolivar and Backman, 1979.
MC1061	*hsdR mcrB araD*139 Δ(*araABC-leu*)7679 Δ*lac*X74 *galU galK rpsL thi*	Meissner *et al.*, 1987
XL1-blue	*rec*A1 *lac*$^-$ *end*A1 *gyr*A96 *thi hsd*R17 *supE*44 *rel*A1 [F' *proAB lac*I^Q *lacZ*ΔM15 Tn10]	Stratagene Manual.

Table 2.1: Bacterial strains used in this study.

2.1.4. Yeast Strains

The yeast strains used in this study are listed in Table 2.2 and were *Saccharomyces cerevisiae* throughout. Whenever possible, strains quoted in the literature were obtained from the authors, and the reference describing their construction is given. A collection of all up to date identified *vps* mutant type alleles were obtained from Tom Stevens, Eugene, Oregon, USA, and are not necessarily all published.

2.1.5. Plasmids

All plasmids used in this study were maintained in either HB101 or MC1061. HB101 was always used to maintain yeast plasmids or plasmids containing yeast genomic DNA sequences, since the *recA*$^+$ host MC1061 can lead to recombination between repeated sequences. Plasmids and their most prominent features are listed in Table 2.3.

Strain	Genotype	Reference
05149	*MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL vps5*	Robinson *et al.*, 1988.
05201	*MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL vps18*	Robinson *et al.*, 1988.
0531	*MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL vps33*	Robinson *et al.*, 1988.
0552-11	*MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL vps11*	Robinson *et al.*, 1988.
0570	*MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL vps16*	Robinson *et al.*, 1988.
0572	*MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL vps17*	Robinson *et al.*, 1988.
14121-26	*MAT***a** *leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL vps1*	Robinson *et al.*, 1988.
15113-8	*MAT***a** *leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL vps8*	Robinson *et al.*, 1988.
B-6164	*MATα cyc1-367 arg4-17 osm2*	Singh and Sherman, 1978.
D-597-6D	*MAT***a** *cyc1-1 trp1-1 aro7-1 leu2-1 osm1*	Singh and Sherman, 1978.
DBY1373	*MAT***a** *his4 ura3-52 tub2-104*	Thomas *et al.*, 1985.

Strain	Genotype	Reference
DBY1691	*MAT***a** *his4-619 act1-1*	Novick and Botstein, 1985.
MWY100	*MAT***a** *ade1*	M.D. Watson, lab strain.
MWY101	*MAT*α *ade1*	M.D. Watson, lab strain.
RH1330	*MAT***a** *aro3-2 aro4-1*	M. Künzler, Zürich.
RH1610	*MAT***a** *Δura3 aro2::URA3*	M. Künzler, Zürich.
S5	*MAT***a** *ade1 ade2 ura1 his7 tyr1 lys7 gal1 gut1*	Sprague and Cronan, 1977.
S22	*MAT***a** *ade1 ade2 ura1 his7 tyr1 lys7 gal1 gut2*	Sprague and Cronan, 1977.
SEY6210	*MAT*α *leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL*	Robinson *et al.*, 1988.
SEY6211	*MAT***a** *leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL*	Robinson *et al.*, 1988.
SF838-1D	*MAT***a** *ade6 his4-519 ura3-59 leu2-3 leu2-112 pep4-3 gal*	T.H. Stevens, lab strain.
vps1 to *47*		T.H. Stevens

Table 2.2: Yeast strains used in this study.

Plasmid	Comments	Reference
pBluescript™	bla^r	Stratagene Manual.
pCKR108	bla^r, carboxypeptidase Y fusion plasmid	C.K. Raymond, Eugene, Oregon.
pCYI433	bla^r, *URA3*	Bankaitis *et al.*, 1986.
pEXP1-3	bla^r, lacZ promoter expression vector	Muchmore *et al.*, 1989.
pRS316	bla^r, *URA3*	Sikorski and Hieter, 1989.
pUC18/19	bla^r	Messing, 1983.
YCp50	tet^r, bla^r, *URA3*	Rose *et al.*, 1987.

Table 2.3: Plasmids used in this study.

2.1.6. Buffers and Stock Solutions (Listed in Alphabetical Order)

Amino Acids Stock Solutions

Stock solutions of individual amino acids or amino acid mixtures were prepared at 2 mg/mL and then autoclaved, except for tryptophan, which was sterile filtered. They were used at a final concentration of 20 μg/mL as supplements of yeast minimal medium as required.

Antibiotic Stock Solutions

Antibiotic stock solutions were prepared and used as shown in Table 2.4. All stocks were kept at -20°C.

Antibiotic	Stock	Solvent	Final Concentration
Ampicillin	25 mg/mL	70% Ethanol	50 µg/mL
Chloramphenicol	20 mg/mL	100% Ethanol	10 µg/mL
Tetracyclin	5 mg/mL	50% Ethanol	10 µg/mL

Table 2.4: Antibiotic stock solutions and their effective final working concentration.

Blocking Buffer

Gelatin 10 g
10x Tris.HCL-Salt 100 mL
in 1 L dH_2O.

Carbonate Buffer pH 9.2

$NaHCO_3$ 2.1 g
in 1 L dH_2O. The solution was adjusted to pH 9.2 with 1 M NaOH.

Coomassie Blue Stain Solution

Coomassie Brilliant Blue 0.2% (w/v)
Methanol 50% (v/v)
Glacial Acetic Acid 7% (v/v)
in aqueous solution.

Ethidium Bromide Stock

Ethidium Bromide 50 mg
in 10 mL dH_2O. The solution was stored in the dark.

Gel Destaining Solution

Methanol 40% (v/v)
Glacial Acetic Acid 10% (v/v)
in aqueous solution.

6x Gel Loading Buffer (Agarose Gel Electrophoresis)

Bromophenol Blue	25 mg
Xylene Cyanol	25 mg
Sucrose	4 g

in 10 mL dH_2O.

10x PAGE Buffer

Glycine	141 g
Tris.HCl	30 g
SDS	10 g

in 1 L dH_2O.

20x PBS (Phosphate Buffered Saline)

NaCl	160 g
KCl	4 g
Na_2HPO_4	28.8 g
KH_2PO_4	4.8 g

in 1 L dH_2O. The solution was adjusted to pH 7.4 with HCl.

Protein Sample Buffer

Glycerol	1 mL
10% SDS	4 mL
0.5 M Tris.HCl pH 6.8	500 µL
250 mM EDTA	40 µL
0.05% bromophenol blue	500 µL

in 10 mL dH_2O. β-mercaptoethanol was added prior to use to 5% (v/v).

50x TAE Buffer

Tris.HCl	242 g
EDTA	18.5 g
Glacial Acetic Acid	57.1 ml

in 1 L dH_2O

10x TBE Buffer

Tris.HCl	121.1 g
Boric Acid	51.35 g
EDTA	3.72 g

in 1 L dH_2O.

TE Buffer pH 8.0 (10 mM Tris.HCl, 1 mM EDTA)

1 M Tris.HCl pH 8.0	10 mL
250 mM EDTA pH 8.0	4 mL

in 1 L dH_2O.

Transfer Buffer

Tris.HCl	6.06 g
Glycine	28.8 g
Methanol	400 mL

in 2 L dH_2O.

5x Tris-Glycine Buffer

Tris.HCl	15.1 g
Glycine	94 g
SDS	5 g

in 1 L dH_2O.

10x Tris-Salt Buffer

Tris.HCl	24.2 g
NaCl	90.0 g

in 1 L dH_2O. The solution was adjusted to pH 7.2 with HCl.

Tween-20 Precoat Buffer

1 M NaH_2PO_4 pH 7.5	10 mL
NaCl	8.2 g
5% (v/v) Tween-20	10 mL

in 1 L dH_2O.

2.1.7. Growth Media (Listed in Alphabetical Order)

All growth media described below can be used as liquid media, or can be solidified by the addition of 2% agar.

Luria-Bertani Broth (Luria Broth)

Bacto-Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

in 1 L dH_2O.

Plate Agar (Sphaeroplast Transformation)

Sorbitol	218.6 g
Yeast Nitrogen Base w/o amino acids	6.7 g
Glucose	20 g
Amino acids	20 µg/mL
Agar	20 g

in 1 L dH_2O.

Presporulation Agar

Yeast Extract	10 g
Nutrient Broth	30 g
Glucose	50 g
Adenine	50 mg
Uracil	5 mg
Agar	20 g

in 1 L dH_2O.

Regeneration Agar (Sphaeroplast Transformation)

Sorbitol	218.6 g
Yeast Nitrogen Base w/o amino acids	6.7 g
Glucose	20g
Amino acids	20 µg/mL
Agar	30 g

in 1 L dH_2O

SOB Medium

Tryptone	20 g
Yeast Extract	5 g
NaCl	0.6 g
KCl	0.5 g

in 1 L dH_2O. Autoclave and add 10 mL 2 M Mg^{2+} stock (1 M $MgCl_2$ and 1 M $MgSO_4$, filter sterilized) just prior to use.

Sporulation Agar

Yeast Extract	1 g
Glucose	0.5 g
Potassium acetate	10 g
Agar	20 g

in 1 L dH_2O. Essential amino acids were added after autoclaving to a final concentration of 20 μg/mL.

Superbroth

Bacto-Tryptone	33.0 g
Yeast Extract	20 g
NaCl	7.5 g
Glucose	1.0 g

in 1 L dH_2O. The medium was adjusted to pH 7.0.

2x TY broth

Trypticase	16 g
Yeast Extract	10 g
NaCl	10 g

in 1 L dH_2O. The medium was adjusted to pH 7.2 to 7.4.

Wickerham's Minimal Medium (Wickerham, 1946)

Glucose	10 g
Proline	5 g
Na_2SO_4	0.71 g
1 M KPO_4 pH 5.7	10 mL
10x Salts	50 mL
1,000x Vitamins	0.5 mL (added after autoclaving)
10,000x Minerals	50 µL
10 mg/mL Amino acids	1 mL

in 500 mL dH_2O.

10x Salts (two components)

Solution 1:	KH_2PO_4	4.38 g
	K_2HPO_4	0.63 g
	in 250 mL dH_2O.	
Solution 2:	$MgSO_4.7H_2O$	2.5 g
	NaCl	0.5 g
	$CaCl_2.2H_2O$	0.5 g
	in 250 mL dH_2O.	

Equal volumes of solutions 1 and 2 were mixed just prior to use to yield 10x salts.

1,000x Vitamins

Biotin	0.2 mg
Ca-panthothenate	40 mg
Inositol	200 mg
Niacin	40 mg
p-aminobenzoic acid	20 mg
Pyridoxine.HCl	40 mg
Thiamine.HCl	40 mg
Riboflavin	20 mg

in 100 mL dH_2O. The solution was filter sterilized and stored at -20°C.

10,000x Minerals

Boric acid	0.5 g
$MnSO_4$	0.4 g
$ZnSO_4$	0.4 g
$FeCl_2.6H_2O$	0.2 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.2 g
NaI	0.1 g
$CuSO_4.5H_2O$	0.04 g
Citric Acid	1 g

in 100 mL dH_2O.

Yeast Minimal Medium

Yeast Nitrogen Base w/o amino acids	6.7 g
Glucose	20 g

in 1 L dH_2O. Required amino acids were added after autoclaving to the desired concentration.

YPD(A)

Yeast Extract	10 g
Bacto Peptone	20 g
Glucose	20 g
(Adenine	20 mg)

in 1 L dH_2O.

YPSA Bromocresol Purple Medium

Yeast Extract	10 g
Bacto Peptone	20 g
Sucrose	20 g
0.4% Bromocresol purple in ethanol	5 mL

in 1 L dH_2O. Just prior to inoculation, 0.01 mg antimycin A in ethanol was added per mL medium.

2.2. General Methodology

2.2.1. Sterile Working Practices

A microbiological, sterile working practice was maintained throughout. All media or materials used in the maintenance or recovery of cultures, transformants, etc. were sterilized by diverse suitable means. If a laminar-flow hood was not available, it was attempted to keep the surrounding free of possible contaminants by repeated wiping of bench surfaces with 70% ethanol, flaming the necks of culture flasks, and immediate sealing of culture vessels after inoculation.

2.2.2. Sterilisation Techniques

All media, buffers, solutions, glassware and plasticware that were resistant to heat treatment were autoclaved at 15 p.s.i., 123°C, for 20 minutes. Heat sensitive solutions were filter sterilized by passing through a 0.22 μm nitrocellulose membrane filter. Heat sensitive plasticware was left overnight in 70% ethanol and dried in a laminar-flow hood. If sterility was an absolute necessity (e.g. electroporation cuvettes), items were irradiated with γ-rays from a ^{60}Co source for 24 hours (4 Mrad).

2.2.3. Polylysine Coating of Microscope Slides

Microscope slides were coated by covering each slide with 1 mg/mL polylysine. After 20 seconds, the slides were washed six times with dH_2O and allowed to air dry. For welled slides 20 μL 1 mg/mL polylysine was added to each well.

2.2.4. Treatment of Dialysis Tubing

Dialysis tubing was boiled in a solution containing 2% (w/v) Na_2CO_3 and 1 mM EDTA (pH 8.0) for 10 minutes. The tubing was rinsed five times in sterile dH_2O,

followed by another 20 minutes autoclaving. Finally, the tubing was rinsed several times in sterile dH_2O and then stored at 4°C in TE buffer.

2.2.5. Maintenace of Stock Cultures - Bacterial Strains

Strains harbouring no plasmids or selectable markers were cultured in Luria broth or on Luria agar plates without antibiotics. Strains containing plasmids and/or selectable markers were grown in medium under selective conditions. A master stock was maintained in 40% (v/v) glycerol at -84°C. For glycerol stocks, 10 mL Luria broth was inoculated with a colony from the respective strain and incubated at 37°C overnight with agitation. The culture was centrifuged at 3,000 g for 5 minutes, the pellets resuspended in 1 mL Luria broth and transferred to a 2 mL glass or plastic vial containing 1 mL 80% (v/v) sterile glycerol. The vial was frozen in liquid nitrogen, and placed in a freezer at -84°C.

2.2.6. Maintenance of Stock Cultures - Yeast Strains

A stock culture of all yeast strains was maintained in glycerol at -84°C. A single colony of each yeast strain was used to inoculate 10 mL YPDA. The culture was grown to saturation at 30°C with agitation for 48 to 72 hours, dependent on the growth rate of the strain concerned. The cells were sedimented by centrifugation at 1,500 g for 10 minutes, resuspended in 0.75 mL YPDA and added to 1.25 mL 80% (v/v) glycerol in a 2.5 mL glass vial. The glycerols were frozen in liquid nitrogen and stored at -84°C until further use. Great care had to be taken when material was taken from the glycerol not to let the glycerol thaw, since yeast glycerols could not be frozen a second time after the initial freezing.

2.2.7. Measurement of Growth - Phage Titration

20 µL phage suspension was diluted with 180 µL SM buffer (5.8 g/L NaCl, 2.0 g/L $MgSO_4$, 50 mL/L 1 M Tris.HCl pH 7.5, 5 mL/L 2% (w/v) gelatin) in a serial

dilution series until the desired dilution was reached. After the addition of 100 µL appropriate log-phase host cells to 100 µL of the above dilution, phage and host cells were incubated at 37°C for 20 minutes. An additional 200 µL of host cells were added, and the suspension was mixed with 3 mL top agar (same as Luria-agar, except with 0.7% (w/v) agarose) at 42°C and poured immediately onto an agar plate. The plates were incubated at 37°C overnight. The phage concentration in plaque forming units (p.f.u.) could be calculated by the formula: p.f.u. / mL = plaques/plate x 1/dilution x 100.

2.2.8. Measurement of Growth - Bacteria

Bacterial growth was either determined spectrophotometrically by measuring the OD_{600} if a correlation between the number of bacteria and the optical density was known, or by viable cell counts. For the latter, a known volume of the culture was diluted down in 1x PBS in decimal steps, and 100 µL of each dilution was plated out in duplicates onto Luria agar plates under the correct selective conditions. The plates were incubated at 37°C for 12 hours, and the plates showing between 15 and 200 colonies were used for the assessment of the number of bacteria present in the original culture.

2.2.9. Measurement of Growth - Yeast

Total cell counts of yeast cultures were done with a Coulter® Multisizer II fitted with a 37 µm aperture and adjusted to take account of all particles between 0.748 and 9.957 µm (window option). A sample of the yeast culture was diluted 20 times with Isoton® electrolyte solution, and two counts were done per sample, subtracting the blank value. The cell number could be calculated as follows:

Total Cell Count/mL = Cell Count (for 25 µL) x 40 x Dilution Factor

Viable counts were done as described for bacteria, with the only difference that dH_2O was used for dilutions, and YPDA agar plates to plate out the dilutions.

2.3. Classical Yeast Genetics

2.3.1. Drop-overlay Complementation of Auxotrophic Haploid Yeast Strains

Complementation of yeast strains of opposite mating type was done in a drop-overlay complementation test as described in Spencer *et al.*, 1989. Complementation, that is the restoration of the wild type phenotype of two complementing mutations in diploid cells, is an indication that the mutations are affecting different genes. If two mutations fail to complement, it indicates that the two mutations have affected the same gene, or one affects a regulatory site for the other gene, or at least one mutation yields an inhibitory gene product.

All auxotrophic haploid strains of opposite mating types having complementary nutritional requirements were grown in 5 mL YPDA at 30°C for 48 hours with agitation. 100 µL aliquots of each culture were taken and added to 900 µL dH_2O. After centrifugation at 12,000 g in a microfuge for 5 minutes, the pellets were washed once with 1 mL dH_2O, and were resuspended in 100 µL dH_2O each. 5 µL aliquots of each **a** strain were dotted onto yeast minimal medium agar plates supplemented with all essential amino acids common to both **a** and α strains, leaving out those which are required by only one strain. After the plates were sufficiently dry, 5 µL aliquots of each α strain were applied onto all different spots of **a** strain mutants. 5 µL aliquots of all strains were also individually dotted onto the selective yeast minimal medium agar plates as controls. Plates were dried at room temperature and subsequently incubated at 30°C for 4 days. Viable auxotrophy-complementing diploids will be able to grow on the selective medium, whereas haploids will not grow since one or several nutritional requirements were missing. The patches of diploid cells could be replica-plated to screen for complementation of mutations like $NaCl^r$ growth, or growth at high temperature of *ts* strains on other selective media or conditions.

2.3.2. Sporulation of Diploids and Random Spore Isolation

Diploids heterozygous for the *ADE* locus were patched out onto presporulation agar and were incubated at 30°C for 48 hours. All the material from the patch was scraped off the plate and patched out onto a sporulation agar plate. The plate was incubated at 30°C for 5 days, or until about 95% of all diploids formed tetrads as detectable by light microscopic analysis. A loopful of the sporulated diploid (about the size of a matchhead) was resuspended in 200 µL glusulase. The suspension was incubated at 30°C for 2 hours with agitation. Sterile glass beads were added to about half the volume of the digest, the mix was vortexed vigorously for 1 minute and 1 mL sterile water was added. The sample was diluted down to 1×10^{-4} in a decimal dilution series. 100 µL aliquots of each dilution was plated out onto YPD agar plates, which were incubated at 30°C for 72 hours. All red colonies (representing *ade* haploids) or small white colonies (generally representing *ADE* haploids) were picked and purified in a fractional streak. The isolated haploids were tested for various markers and also for the mating type, so that mutants of opposite mating type could be isolated.

2.3.3. Testing for Mating Type

Testing of mating type was done as described in Spencer *et al.*, 1989, using strains MWY100a and MWY101α as mating type testers. These strains carry a mutation in the *ADE1* locus, but are wild type for all other genetic markers concerned. This allows the testing for mating type of all but *ade1* strains on unsupplemented yeast minimal medium.

The two tester strains were streaked onto a yeast minimal medium plate parallel to each other, and the strain to be tested was streaked perpendicular to each of the two streaks. The plates were incubated at 30°C for 4 days, and colonies that were able to grow only on one overlapping cross were recorded as the mating type opposite to the tester strain.

2.3.4. EMS - Mutagenesis

Yeast strains were mutagenized with ethanemethylsulphonate (EMS) at 50% killing after the method of Fink, G.R., 1970.

A medium size colony of the yeast strain to be mutagenized was used to inoculate 10 mL YPDA, which was incubated at 30°C for 24 hours with agitation (140 rpm). The cells were centrifuged at 1,500 g for 10 minutes, the pellets were resuspended in 10 mL dH_2O, and it was centrifuged as above. The cells were resuspended in 10 mL 0.1 M sodium phosphate buffer pH 8.0, and 0.3 mL EMS was added to each tube. The tubes were vortexed vigorously to disperse the EMS, and the suspension was incubated at 30°C without agitation for exactly 50 minutes. The cells were centrifuged at 1,500 g for 10 minutes, were resuspended in 10 mL dH_2O and then transferred to a new sterile tube. This washing process was repeated twice, and the cells were resuspended in 10 mL dH_2O after the third wash. The cell suspension was diluted down to 1×10^{-5} in a serial dilution, and 100 µL aliquots were plated out onto YPDA agar plates. The plates were incubated at 30°C for 48 hours, and then could be replica-plated onto the selective medium or screened in dot blot hybridization tests.

2.3.5. UV - Mutagenesis

Yeast strains were also subjected to mutagenesis by UV irradiation to induce a potentially different set of mutants to the ones generated by EMS mutagenesis. Since the degree of killing strongly depends on the light source and wavelength used, a dose-response curve had to be constructed to allow mutagenesis at 5% survival. For that purpose a single yeast colony from a fresh YPDA agar plate was used to inoculate 10 mL YPDA. The culture was incubated at 30°C and agitation (140 rpm) for 48 hours to saturation. The cells were harvested by centrifugation at 1,500 g for 10 minutes, the pellet was resuspended in 10 mL dH_2O and cooled to 4°C to inhibit further cell division. The suspension was given into a 9 cm diameter petri-dish fitted with a magnetic stirrer (70 mm long; 9 mm diameter) which rotated at a speed-setting of 4 of a Gallenkamp Magnetic Stirrer Hotplate 400. The set-up was placed underneath a UV

lamp (CAMAG universal UV lamp TL900; 254 nm, 10 cm from the surface of the suspension). Irradiation of the cells was started by removing the lid from the petri-dish, and 100 µL samples were taken in 15 second intervals for 450 seconds. The samples were diluted down to $1x10^{-6}$ in a decimal dilution series, and 100 µL aliquots of the $1x10^{-6}$, $1x10^{-5}$, and $1x10^{-4}$ dilution were plated out in duplicates onto YPDA agar plates, which then were incubated at 30°C for 48 h. The percentage of survivors was plotted against the time (dose) of UV irradiation, and the dose which leads to 95% killing of the yeast cells originally present was used in subsequent mutagenesis events under the same conditions as described above.

2.3.6. Selection for $NaCl^s$ mutants

Mutagenized yeast cultures were diluted with sterile dH_2O in decimal steps and plated out onto YPDA agar plates. After incubation at 30°C for 48 hours, all plates which showed between 20 and 200 colonies were replica plated onto YPDA plates supplemented with 1.5 M NaCl using a wooden block covered with a sterile piece of velvet. The plates were sealed with Para-film™ and incubated at 30°C for 7 days. Master and replica were compared, and all colonies which were able to grow on YPDA but not on YPDA + 1.5 M NaCl were thought to be sensitive to high osmotic conditions, and were picked. The colonies were further purified in two successive fractional streaks, and after repeated testing for the inability to grow on YPDA + NaCl as before, stocks were produced.

2.4. DNA Manipulation

All methods described in the following section were adopted directly from Sambrook *et al.*, 1989, or were modified protocols, which worked equally well under the conditions employed. Any of the following protocols which originated from another source are quoted from the literature.

2.4.1. Plasmid DNA Preparation from *E. coli* - Large Scale

10 mL of Luria broth containing the appropriate antibiotic was inoculated with a colony of the bacterium harbouring the plasmid to be isolated, and then incubated at 37°C for 16 hours with agitation. The culture was then used to inoculate 500 mL of superbroth containing antibiotic at the correct concentration, and then incubated at 37°C and 200 rpm for 6 hours, or until the OD_{600} reached a value of 0.9 to 1.0, using superbroth as a blank. 100 mg solid chloramphenicol per 500 mL culture was added and the culture incubated for another 16 hours at 37°C and 200 rpm. The chloramphenicol amplification step described here was optional, and unnecessary for high copy number plasmids (e.g. pUC derivatives). If chloramphenicol was omitted, the cultures were incubated for 18 hours under the same conditions as described before. Cells were harvested by centrifugation in 250 mL centrifuge tubes at 3,500 g and 4°C for 1 minute. The pellet was washed once with 250 mL 25 mM Tris.HCl pH 8.0, 10 mM EDTA without resuspending the pellet. It was centrifuged under the same conditions as described above. The pellet of a 250 mL aliquot of the original culture was resuspended in 5 mL 15% (w/v) sucrose, 50 mM Tris.HCl pH 8.0, 50 mM EDTA (STE), and aliquots of 20 mL of this suspension were transferred to 100 mL centrifuge tubes. The tubes were frozen at -84°C for 30 minutes and were left at room temperature until completely thawed. 30 mL STE and 4 mL of a 10 mg/mL lysozyme solution in STE were added to each tube, which then were incubated at 37°C for 10 minutes, and a further 15 minutes on ice. 12 mL 5 M potassium acetate were added to each tube and left on ice for 15 minutes. 6 mL 10% (w/v) SDS each was added, and the tubes were gently mixed at room temperature for 10 minutes by slow inversion before they were left on ice for 60 minutes. The tubes were balanced and centrifuged at 12,000 g and 7°C for 20 minutes. The supernatant was aspirated and pooled. Solid NaCl was added to 36 g/L supernatant and when completely dissolved, 50% (w/v) PEG-6000 was added to reach a final concentration of 10%. After mixing, this solution was left on ice for 14 to 16 hours. The white sediment formed was resuspended and then centrifuged at 8,000 g and 4°C for 15 minutes. The pellets were slowly dissolved in a total of 18 mL TE (for

500 mL plasmid prep), and solid CsCl was added to a final concentration of 0.94 g per g sample. It was centrifuged at 2,500 g and 4°C for 20 minutes to float any residual PEG. PEG on top of the solution was removed, and 10 mg/mL ethidium bromide solution was added to give a final ratio of 10 µL/mL sample. The sample was introduced into quickseal ultracentrifuge tubes, which after balancing were sealed and centrifuged at 200,000 g (VTi70 rotor) at 15°C for 20 hours in a Sorvall OTP65B Ultracentrifuge. The tubes were carefully removed, DNA was visualized under U.V., and the lower visible band (plasmid DNA) was taken up with a syringe, after piercing the top of the tube. If high purity of the plasmid preparation was essential, the samples containing the plasmid were given into a fresh 15 mL quickseal tube and then topped up with a 0.94 g/mL CsCl plus 30 µL/mL ethidium bromide solution. The tubes were centrifuged as before, and the plasmid DNA was carefully taken off the gradient with a syringe. The sample was extracted with an equal volume CsCl and TE buffer saturated isopropanol about 4 to 5 times to remove the ethidium bromide, and it was dialysed against a total of 4 L TE with four changes of buffer for 12 hours between each buffer change. The concentration of the plasmid DNA was determined, and the DNA was stored at -84°C.

2.4.2. Plasmid DNA Preparation from *E. coli* - Mini Preps

A method for the isolation of small amounts of plasmid DNA (4 to 10 µg) from *E. coli* using silica fines was employed throughout (Amersham).

5 mL Luria broth containing the appropriate antibiotic were inoculated with a single bacterial colony, and incubated at 37°C overnight with vigorous agitation (200 rpm). 1.5 mL of the culture were transferred into a microcentrifuge tube and centrifuged for 1 minute at 12,000 g in a microfuge. The supernatant was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 350 µL of a sterile filtered solution containing 8% sucrose, 0.5% (v/v) Triton X-100, 50 mM EDTA, 10 mM Tris.HCl pH 8.0. 25 µL of a freshly prepared lysozyme solution (10 mg/mL lysozyme in TE buffer) was added, and it was vortexed for 3 seconds. The tube was incubated in a boiling waterbath for exactly 90 seconds. It was then immediately

centrifuged at room temperature for 10 minutes, and the pellet was removed from the tube with a sterile toothpick. To the supernatant 0.7 mL NaI solution (90.8 g NaI, 1.5 g Na_2SO_3 in 100 mL dH_2O, filter sterilized, and 0.5 g Na_2SO_3 added after filtering) was added and mixed by gentle vortexing. 20 µL of well resuspended, acid washed silica fines (silica 325 mesh, 50% (v/v) in dH_2O) were added to the tube, mixed by inversion, and the plasmid DNA bound to the glass by incubation at room temperature for 10 minutes. The tubes were centrifuged for 15 seconds at 12,000 g and all of the supernatant aspirated off. The pellet was resuspended in 1 mL 70% (v/v) ethanol:30% (v/v) TE buffer, and then centrifuged as before. The ethanol was aspirated off, the pellets were dried under vacuum for 10 minutes, and the fines then resuspended in 50 µL TE buffer. DNA was eluted by incubating the tube at 37°C for 10 minutes. The fines were pelleted by centrifugation as before, and the plasmid DNA containing supernatant was transferred to a fresh tube. The DNA was now ready for digestion with any restriction endonuclease or transformation without any further purification.

2.4.3. Genomic DNA Isolation from *S. cerevisiae* - Large Scale (Rose *et al.*, 1987)

Two 10 mL YPDA starter cultures were inoculated with a medium size yeast colony, and incubated at 30°C for 36 hours with agitation. The contents of both starter cultures were transferred to 1 L YPDA and then incubated at 30°C and 140 rpm for 16 hours until a cell density of 1 x 10^8 cells/mL was reached. Cells were harvested by centrifugation at 7,500 g and 4°C for 5 minutes. The pellets were resuspended in 50 mL 50 mM EDTA pH 9.5 each, and the cell suspension centrifuged under the same conditions as above. Each pellet was resuspended in 25 mL ice-cold 50 mM Tris.HCl pH 9.5, 2% (v/v) β- mercaptoethanol, pooled into a 250 mL centrifuge tube and left on ice for 15 minutes. The cell suspension was centrifuged as before and the pellet resuspended in 20 mL 1 M sorbitol, 1 mM EDTA pH 8.5 and then 500 µL of a 10 mg/mL lyticase-100 solution in the same buffer was added. The cells then were sphaeroplasted at 37°C with gentle shaking (120 rpm) until more than 95% of the cells burst upon dilution of a 10 µL sample in 1 mL 1% (w/v) sarcosyl (3.5 hours). The

suspension was centrifuged at 12,000 g and 4°C for 15 minutes. The pellet was suspended in 5 mL lysis buffer (0.1 M Tris.HCl pH 9.5, 0.15 M NaCl, 0.1 M EDTA, 2% (v/v) β-mercaptoethanol), and the centrifuge tube was frozen in liquid nitrogen for about 15 to 30 minutes. The frozen sample was thawed at room temperature and transferred to a 100 mL conical flask (total volume approximately 10 mL). 20 mL lysis buffer, pH 9.4, with 4% (w/v) sarcosyl was added, and the lysis mixture was incubated at 45°C for 20 minutes. 20 mL lysis buffer, pH 8.0, with 4% (w/v) sarcosyl was added to the flask, which was then incubated at 70°C for 15 minutes. DNAse free RNAse was added to a final concentration of 0.1 mg/mL, and then incubated at 45°C for 1 hour. Proteinase K (Type XI) was added to a final concentration of 0.8 mg/mL in two aliquots in hourly intervals. The mixture was incubated at 37°C for a total of 2 hours, followed by incubation at 70°C for 15 minutes. 10 mL aliquots of the digest were transferred to 30 mL cortex tubes. 10 mL of a phenol:chloroform mixture (1:1) was added to each tube, the tubes were then sealed with cling film and slowly agitated until a white emulsion formed and no phase boundaries were visible anymore. Phases were separated by centrifugation at 5,000 g and 15°C for 15 minutes. The DNA containing top layer was extracted once more with phenol:chloroform and twice with chloroform. The aqueous fractions were pooled and incubated at 45°C for 2.5 hours to remove traces of chloroform. The DNA was dialysed against 0.15 M NaCl, 10 mM Tris.HCl pH 8.0, 1 mM EDTA at 4°C with continuous stirring. The dialysis buffer was changed twice after 8 hours, and the DNA was pooled and the volume determined. Solid CsCl was added to reach a final concentration of 1 g CsCl/0.8 mL. The solution was filled into quickseal tubes which were topped up with CsCl solution of the same density, balanced and sealed. The tubes were centrifuged at 200,000 g and 15°C in a Sorvall OTP65B centrifuge (VTi50 rotor) for 24 hours. The tubes were perforated with a wide gauge syringe needle, and 1 mL fractions were collected. The fractions were tested for DNA concentration, and the fractions which contained the DNA were pooled and dialysed against TE buffer in dH_2O for 24 hours with three buffer changes. The DNA concentration was determined spectrophotometrically and then stored at -84°C.

2.4.4. Isolation of Plasmid DNA from *S. cerevisiae*

A single step purification protocol for shuttle plasmids from yeasts for transformation into *E. coli* was employed as described by Ward, 1990. The plasmid harbouring yeast strain was grown under selective conditions until saturation, usually 48 hours at 30°C and 140 rpm, and cells were harvested by centrifugation of a 1.5 mL aliquot at 12,000 g for 30 seconds. The pellet was suspended in 100 µL of LTTE (2.5 M LiCl, 50 mM Tris.HCl pH 8.0, 4% Triton X-100, 62.5 mM EDTA), 100 µL phenol:chloroform (1:1), and 0.2 g acid washed glass beads were added. The mixture was vortexed at top speed for 2 minutes, and phases were separated by centrifugation at 12,000 g for 1 minute. The upper phase was ethanol precipitated and the DNA was dried under vacuum and redissolved in 20 µL TE buffer. A 1µL aliquot of the DNA could be transformed into *E. coli*.

2.4.5. Determination of the DNA Concentration

The concentration of aqueous nucleic acid solutions was determined spectrophotometrically at a wavelength of 260 nm and 280 nm in a Philips UV/Visible PU8740 Spectrophotometer. An OD_{260} of 1.0 corresponds to about 50 µg/mL dsDNA, 40 µg/mL ssDNA and RNA, and approximately 20 µg/mL for single stranded oligonucleotides. The OD_{260}/OD_{280} ratio could estimate the purity of the DNA/RNA measured. For pure nucleic acid the ratio is approximately 1.8 for DNA, and 2.0 for RNA. If the preparation is contaminated with phenol or proteins, the ratio will be much smaller.

2.4.6. Phenol:Chloroform Extraction of DNA

The method makes use of the fact that proteins and DNA have different partitioning properties in a mixture consisting of an organic (phenol) and aqueous phase.

An equal volume of a 1:1 mixture of phenol:chloroform, which before had to be buffer equilibrated to pH 8.0, was added to the nucleic acid sample to be extracted, and the contents of the tube was gently emulsified. The emulsion was centrifuged at 12,000 g for 30 seconds in a microfuge, and the aqueous phase was transferred to a fresh tube, and the extraction process could be repeated until no precipitate at the interphase was formed. The aqueous phase was transferred to a new tube, an equal amount of chloroform was added, and the tubes were gently mixed until an emulsion was formed. The emulsion was centrifuged at 12,000 g for 30 seconds in a microfuge and the aqueous phase was transferred to a fresh tube. The DNA in the aqueous phase could be ethanol precipitated as described below.

2.4.7. DNA Precipitation from Aqueous Solutions

DNA could be precipitated with isopropanol or with ethanol in the presence of sodium acetate or lithium chloride. For isopropanol precipitation, 0.7 volumes isopropanol were added to the DNA solution and then immediately centrifuged for 30 minutes at 12,000 g and room temperature. The supernatant was aspirated off, and the mostly invisible DNA pellet was washed with 1 mL ice-cold 80% (v/v) ethanol. After further centrifugation for 1 minute, the supernatant was aspirated off and the pellet dried either under vacuum for 1 to 2 minutes or by leaving the tubes inverted at room temperature. For ethanol precipitations, 1/10 volume of 5 M sodium acetate or 8 M LiCl were added to the DNA containing solution, and 2 volumes ice-cold ethanol were added to the solution. Tubes were left on ice for 15 minutes, and DNA was pelleted by centrifugation at 12,000 g for 15 minutes. Pellets were washed and dried as described for isopropanol precipitations. Generally, the addition of monovalent ions could be omitted, if the DNA was in buffer containing high salt concentrations.

2.4.8. Restriction Digests

Restriction digests were carried out using commercial sources of enzymes and their appropriate buffers from the same supplier. A typical digest was sequentially set up as described below:

DNA	X μL
dH_2O	18-X μL
10x Restriction Enzyme Buffer	2 μL
Restriction Enzyme	1 U/μg DNA

Digestion was carried out for 3 hours at the recommended temperature, usually 37°C, except for Sma I where the temperature optimum was 25°C. Double digests were carried out using the Boehringer buffer system, if restriction enzymes were compatible, and both enzymes were added simultaneously to the digest. If enzymes were incompatible in their buffer requirement, digestion was carried out with one enzyme, the digest was phenol:chloroform extracted, and DNA was precipitated and used in a subsequent restriction digest. If larger amounts of DNA were required, the above reaction could be scaled up appropriately.

2.4.9. DNA Molecular Weight Markers

DNA molecular weight markers were prepared by digestion of phage λ DNA with Eco RI, Pst I or Hind III to completion. A typical preparative digest was performed in the following reaction:

λ DNA (300 μg/mL)	166 μL
dH_2O	624 μL
10x Restriction Enzyme Buffer	90 μL
Restriction Enzyme (10 U/μL)	20 μL

Digestion was carried out overnight at 37°C. 100 μL 6x stop dye was added, and the molecular wight marker was dispensed into 100 μL aliquots and stored frozen at -20°C. An equivalent of 20 to 25 μL was loaded onto agarose gels as a size standard.

2.4.10. Dephosphorylation of Termini

Dephosphorylation of 5'-phosphate groups of linearized DNA was carried out with phenol:chloroform extracted and ethanol precipitated DNA in the following reaction:

Linearized DNA	45 µL
10x CIP Buffer	5 µL
Calf Intestinal Alkaline Phosphatase (CIP)	2 µL

Dephosphorylation was carried out at 37°C for 30 minutes. The phosphatase was inactivated by adding 5 µL 0.1 M NTA, pH 8.0 and heating to 75°C for 20 minutes. DNA was twice phenol:chloroform extracted, and DNA was ethanol precipitated and could be used in ligation reactions.

2.4.11. Ligation Reactions

Ligation reactions of cohesive and blunt-ended termini were set up by placing an equivalent of 0.1 µg vector DNA and an equimolar amount of the fragment to be ligated into the vector into a microcentrifuge tube, and adding water to a total volume of 18 µL. The mix was heated up to 45°C for 5 minutes, and then placed on ice. 2 µL 10x ligase buffer (200 mM Tris.HCl pH 7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol, 500 µg/mL BSA, 5 mM ATP) and 1 µL T4 DNA ligase (1 Weiss unit) were added into the tube, and ligation was carried out at room temperature for 2 to 4 hours, or at 4°C overnight. 1 to 10 µL of the ligation reaction could be transformed into competent *E. coli*.

2.4.12. Klenow Filling-in of Cohesive Termini

Klenow filling-in of recessed 3' cohesive termini could be done immediately after restriction digestion without phenol:chloroform extraction. 1 µL of a solution of all 4 dNTP's at a concentration of 1 mM was added to 20 µL of the restriction digest, 1 unit Klenow fragment/µg DNA was added, and the reaction was carried out at room temperature for 15 minutes. The reaction was terminated by adding an equal amount of TE buffer, phenol:chloroform extraction and subsequent ethanol precipitation.

2.4.13. Exo III/Mung Bean Nuclease Deletion

The Exo III/mung bean deletion system™ (Stratagene) has been designed to carry out unidirectional deletions of DNA of predictable sizes. The Exo III nuclease will digest 3' termini from blunt and 5' protruding termini, but not 3' single strand termini. Deletions of an insert in the pBluescipt™ vectors proceeded according to the manufacturer's recommendations, using only reagents supplied with the kit (Bluescript™ Exo/Mung DNA Sequencing System Instruction Manual, Stratagene).

The plasmid construct was double digested to completion with two restriction enzymes. One enzyme creates a 5'-protruding or blunt end, the other 3'-protruding termini. The 5'-protruding or blunt end is between the sequence to be deleted and the 3'-protruding terminus. DNA was phenol:chloroform extracted, ethanol precipitated and dissolved in TE buffer. The Exo III reaction was set up as follows for each time point:

Double digested DNA	5 μg
2x Exo Buffer	12.5 μL
100 mM β-mercaptoethanol (fresh)	2.5 μL
Exonuclease III	1 μL
dH_2O to	25 μL

The reaction was started by adding Exo III, and incubation at the optimal temperature (Table 2.5) to yield the desired deletions. 25 μL of the reaction mixture was removed at the appropriate time, and given into a tube containing 40 μL 5x Mung Bean Buffer and 135 μL dH_2O and then placed on ice. When all aliquots were removed, tubes were heated to 68°C for 15 minutes and then placed on ice. 40 units Mung Bean Nuclease (diluted with 1x Mung Bean Dilution Buffer) was added to each tube, and then incubated at 30°C for 30 minutes. 4 μL 20% (w/v) SDS, 10 μL 1 M Tris.HCl pH 9.5, 20 μL 8 M LiCl, and 250 μL phenol:chloroform were added, mixed by vortexing thoroughly, and then centrifuged at 12,000 g for 1 minute. The aqueous layer was chloroform extracted, 0.5 mL ice-cold ethanol added and the DNA ethanol precipitated as described before. The now blunt ended deletions were self ligated, transformed into *E. coli*, and transformants were screened for the presence of the correct size deletion.

Temperature	Exo III dsDNA to ssDNA conversion
37°C	400 bp/minute
34°C	375 bp/minute
30°C	230 bp/minute
23°C	125 bp/minute

Table 2.5: Progress of Exo III digestion at different incubation temperature.

2.4.14. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in a Pharmacia mini-gel system, or a home made maxi gel apparatus. The open ends of the gel casting apparatus were sealed with autoclave tape, or a casting mold was placed onto a glass plate, and the casting mold was set on a horizontal place and combs inserted. Agarose was added to a measured quantity of 1x TAE buffer, usually to a concentration of 0.7% (w/v), and the suspension was boiled in a microwave oven until all the agarose had dissolved. After cooling down to approximately 60°C, ethidium bromide was added to a final concentration of 0.5 μg/mL and the agarose poured into the mold. Once the gel had set, the comb was removed and the gel placed into the electrophoresis apparatus. 1x TAE buffer containing 0.5 μg/mL ethidium bromide was added so that the surface of the gel was covered to a depth of 1 to 2 mm. DNA samples in 6x sample buffer were loaded and electrophoresis performed at 1 to 5 V/cm until the marker dyes had migrated to appropriate distances. The gel then was placed onto a UV illumination apparatus and photographed using a red-filter and a polaroid camera.

2.4.15. DNA Fragment Isolation

DNA fragments separated in a low melting point agarose gel were excised with a sterile razor blade, heated up to 70°C for 10 minutes and 700 μL NaI (see 2.4.2.)

solution added. After gentle mixing by inversion 10 µL silica fines was added and the DNA was bound and eluted from the fines as described previously (2.4.2.).

2.5. DNA Sequencing

2.5.1. Procedure for Obtaining Single Stranded DNA from Bluescript™ Vectors

A tube containing 10 mL Luria broth supplemented with ampicillin and tetracycline was inoculated with XL1-Blue harbouring recombinant pKS/pSK plasmid. After overnight incubation with agitation at 37°C, a 5 mL equivalent was transferred to 50 mL 2x TY broth containing ampicillin and tetracycline and incubated for 4 to 6 hours until an OD_{600} of 0.3 was reached (about 2.5 x 10^8 cells/mL). Helper phage R408 was added to the log-phase culture at a multiplicity of infection (m.o.i.) of 20:1 (phage:bacteria). The culture was incubated for another 6 to 8 hours at 37°C with moderate (160 rpm) agitation. The culture was clarified by centrifugation at 17,000 g for 20 minutes and the supernatant transferred to a fresh tube. A 10 mL aliquot of the suspension was added to 2.5 mL 3.5 M ammonium acetate pH 7.5, 20% (w/v) polyethyleneglycol solution. It was mixed by inversion and incubated at room temperature for 15 minutes. Tubes were centrifuged at 11,000 g for 20 minutes and the supernatant thoroughly drained off. The pellet was resuspended in 0.5 mL TE buffer, 0.5 mL buffer saturated phenol:chloroform was added and then vortexed for 1 minute. The tube was centrifuged for 5 minutes, the supernatant transferred to a fresh tube and the procedure was repeated until no interphase was left (up to 5 times). 0.5 mL chloroform was added to the aqueous phase of the final phenol:chloroform extraction, vortexed for 1 minute and then centrifuged for 2 minutes. The aqueous phase was transferred to a fresh tube. The ssDNA was precipitated by adding 375 µL 7.5 M ammonium acetate pH 7.5, and 1.5 mL cold 100% ethanol in a 15 mL Corex tube. The mixture was left on ice for 15 minutes and then centrifuged at 4°C for 20 minutes. The

supernatant was aspirated off, the pellet washed with 80% (w/v) ethanol and dried under vacuum. The pellet was redissolved in 100 µL dH_2O, and transferred to a fresh tube.

2.5.2. Preparation of ds Plasmid DNA Template for Sequencing

1.1 mL of an overnight culture in Luria Broth of the strain harbouring the desired plasmid was transferred to a microfuge tube and bacteria were pelleted by centrifugation at 12,000 g for 3 minutes. The pellet was resuspended in 110 µL 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Tris.HCl pH 8.0, 50 mM EDTA, and then 12 µL of a freshly prepared 5 mg/mL lysozyme solution was added and incubated at room temperature for 10 minutes. The tube was placed in a boiling waterbath for 2 minutes, and centrifuged at 12,000 g for 15 minutes. The pellet was removed with a sterile toothpick, and 110 µL isopropanol was added prior to centrifugation for 15 minutes. The pellet was resuspended in 25 µL TE buffer, extracted twice with phenol:chloroform and once with chloroform. The aqueous phase was transferred to a new tube and 20 µL 7.5 M ammonium acetate and 100 µL ice-cold ethanol was added. The tube was placed on ice for 15 minutes and then centrifuged for 20 minutes. The DNA pellet was washed with 1 mL 80% (v/v) ethanol and the pellet dried under vacuum. The DNA was dissolved in 15 µL TE buffer. 5 µL of the solution contains approximately 2 µg DNA.

2.5.3. Alkaline Denaturation of dsDNA Sequencing Templates (Yanisch-Perron *et al.*, 1985)

A 5 µg equivalent of the dsDNA to be sequenced was added to 45 µL 0.2 M NaOH, 0.2 mM EDTA, and denaturation was performed by incubation for 30 minutes at 37°C. The mixture was neutralized by the addition of 5 µL 3 M sodium acetate pH 5.0 and the DNA was precipitated by adding 150 µL ethanol and incubation at -84°C for 15 minutes, or at -20°C overnight. After centrifugation the pellet was washed with 1 mL 70% (v/v) ethanol and dried under vacuum. It was resuspended in 7 µL dH_2O and used in the sequencing reaction described below.

2.5.4. DNA Sequencing Reaction

The sequencing reactions were performed in accordance with the Sequenase Version 2.0™ protocol (Unites States Biochemical, Cleveland, Ohio, USA; 5th edition).

In brief, for a typical non-extended sequencing reaction, the following were combined in a microcentrifuge tube:

Template DNA (1 μg ssDNA; 5 μg dsDNA)	7 μL
Reaction Buffer	2 μL
Primer (1 pM/μL)	1 μL

It was important to use an approximate equimolar ratio of template and primer for best results. All dsDNA to be sequenced had to be alkaline denatured and ethanol precipitated to allow primers to bind. The tube was incubated at 65°C for 2 minutes and then cooled slowly to room temperature over a period of 30 minutes. This was done by placing the tubes in a beaker containing 5 mL water of 65°C, and leaving the beaker at room temperature. Once the temperature was below 30°C, tubes were placed on ice. The following components were added to the same tube:

0.1 M dithiothreitol	1.0 μL
1x Labeling Mix (dGTP)	2.0 μL
[α-^{35}S]dATP (10 μCi/μL)	0.5 μL
Diluted Sequenase/Pyrophosphatase	2.0 μL

1 μL Sequenase Version 2.0 T7 DNA Polymerase and 0.5 μL pyrophosphatase were diluted in 6.5 μL enzyme dilution buffer and left on ice no longer than 15 minutes. Once the Sequenase was added to the labelling reaction it was mixed and incubated at room temperature for 2 minutes. Four 3.5 μL aliquots of the labelling reaction were transferred into four tubes labelled G, A, T and C, containing 2.5 μL of the ddGTP, ddATP, ddTTP and ddCTP termination mixes, respectively. Tubes containing the termination mixes were pre-warmed to 38°C for at least 2 minutes in a heating block before the labeling reaction was added. The termination reaction was carried out for 5 minutes at 38°C. 4 μL of stop solution was added to each of the four termination reactions. Samples could be stored at -20°C for up to one week, or could be loaded onto a pre-heated sequencing gel. Before loading the gel, samples were heated up to 80°C for 2 minutes and loaded immediately.

2.5.5. Denaturing Gel Electrophoresis

A Bio-Rad® 21 x 50 cm Sequi-Gen Nucleic Acid Sequencing Gel System was used to pour and run sequencing gels. Before pouring the gel, the glass plates were cleaned with ethanol and acetone, and one plate was siliconized with Repelcote and polished. The gel apparatus was assembled and ready for casting.

To make the gel, 42 g urea, 10 mL 10x TBE buffer and 40 mL dH_2O were heated to 65°C in a water bath, until the urea was completely dissolved. 12.5 mL of a 40% (w/v) acrylamide stock solution (380 g/L acrylamide, 20 g/L Bis-acrylamide) for a 5% gel was added, the volume made up to 100 mL and then filtered through a 22 µm Satorius membrane filter. 20 mL of the unpolymerized gel solution was transferred to a beaker, 140 µL 25% (w/v) ammonium persulphate solution and 100 µL TEMED were added and the solution was poured into the casting tray onto filter paper, and the gel assembly was placed on top. After 5 minutes, the gel assembly was placed in a horizontal position. To the remaining 80 mL of unpolymerized gel solution, 117 µL 25% (w/v) ammonium persulphate and 90 µL TEMED were added, the gel was poured and an appropriate comb was inserted between the glass plates. After the gel had polymerized it was pre-run at 30 mA to a temperature of 55°C, using 1x TBE as the electrophoresis buffer. Before loading the samples, the wells were washed out with buffer. The gel was run at 30 mA and 55°C until the bromophenol blue marker was eluted off the sequencing gel.

2.5.6. DNA Sequence Analysis

Primary DNA sequence analysis was carried out by entering the DNA sequence into the DNA-Strider program (Marck, 1988), assembling the complementary strand, and analysing restriction sites, creating graphical maps of the sequenced fragments, and translating all six hypothetical reading frames, as described in the manual. Additionally, the DNA sequence was down-loaded to the Daresbury SERC SEQNET facility and an analysis using the UWGCG (Devereux *et al.*, 1984) package was performed. This included database searches, determination of DNA sequence features, such as

terminators, start and stop codons, and codon preference comparisons. The most likely start codon was chosen, using a codon bias table, as described in Cavener and Ray, 1991, and searching for upstream regulatory elements at the appropriate distance. Judged from the codon preference comparison, the most probable open reading frame was translated into protein code and further analyzed as described below.

2.5.7. Deduced Protein Sequence Analysis

The deduced protein sequence was compared to previously published sequences, searching the OWL non-redundant database on SEQNET with NEWSWEEP and a daily updated GenEMBL database with TFASTA and FASTA, alignment programs based on the algorithm of Lipman and Pearson, 1985, at a K-tuple of 1. Possible consensus sequences were determined by searching the LUPES and UWGCG consensus sequence libraries for matches. Secondary structure predictions were produced using the UWGCG program (all available on SEQNET).

2.6. Transformation

2.6.1. Transformation of *E. coli* - Ultra High Efficiency

A modified version of the method described by Kushner *et al.*, 1978, for ultra high efficiency transformation (e.g. for libraries) was routinely used to transform *E. coli*.

Cells were grown up in 10 mL Luria broth directly from a glycerol or from an overnight culture to a cell density of 2.0 x 10^8 cells/mL. 1.4 mL aliquots of the culture were centrifuged in a cooled microfuge at 12,000 g for 30 s and the pellets gently resuspended in 500 µL 10 mM MOPS pH 7.0, 10 mM RbCl. The suspension was centrifuged for 15 s at 12,000 g and the pellets resuspended in 500 µL 100 mM MOPS pH 6.5, 10 mM RbCl, 50 mM $CaCl_2$ and held on ice for 90 minutes. Cells were pelleted in a 10 second spin and were resuspended in 150 µL of the same buffer. 3 µL DMSO

and up to 100 µL of the DNA solution (1 - 250 ng) were added consecutively. The transformation mixture was kept on ice for 60 minutes. The tubes were mixed by inversion, heat shocked at 55°C for 30 seconds and kept on ice for 1 minute. 1 mL pre-warmed Luria broth was added to each tube and then incubated at 37°C for 1 hour. 100 µL aliquots of a decimal dilution series of the transformed cells were plated out onto pre-warmed antibiotic containing Luria agar plates.

2.6.2. Transformation of *E. coli* - RbCl Competent Cells (Hanahan, 1985)

30 mL SOB was inoculated with a fresh colony of the strain to be transformed and the culture incubated overnight at 37°C with moderate agitation (160 rpm). 8 mL of the overnight culture were added to 200 mL SOB in a 2 L flask and incubated at 37°C to an OD_{550} of 0.3. The culture was collected in four 50 mL polypropylene centrifuge tubes and left on ice for 15 minutes. Cells were centrifuged at 4°C and 3,000 g for 5 minutes and the supernatant was aspirated off thoroughly. Pellets were gently resuspended by mild vortexing of each in 16 mL sterile filtered and pre-chilled transformation buffer 1 (12 g/L RbCl, 9.9 g/L $MnCl_2.4H_2O$, 30 mL/L 1 M potassium acetate pH 7.5, 1.5 g/L $CaCl_2.2H_2O$, 150 g/L glycerol, pH 5.8 with acetic acid) and incubated on ice for 15 minutes. Cells were pelleted as before and each pellet resuspended in 4 mL ice-cold sterile filtered transformation buffer 2 (20 mL/L 0.5 M MOPS pH 6.8, 1.2 g/L RbCl, 11.0 g/L $CaCl_2.2H_2O$, 150 g/L glycerol), distributed in 600 µL aliquots into sterile, ice-cold microcentrifuge tubes and frozen in liquid nitrogen. Cells were stored at -84°C until required.

For transformation, tubes were thawed on ice and 300 µL aliquots of competent cells were transferred to pre-chilled tubes. 20 µL of the DNA to be transformed was added whilst stirring the cells. The suspension was incubated on ice for 45 minutes and then heat shocked at 42°C for 45 seconds without shaking the tube. The tubes were placed on ice for 5 minutes, 1 mL Luria broth added and transformed cells were plated out onto selective medium after 1 hour incubation at 37°C. Transformants were obtained after 12 to 36 hours incubation at 37°C, dependent on the strain used.

2.6.3. Transformation of *S. cerevisiae* Sphaeroplasts

The method described by Beggs, 1978, was used to transform yeast sphaeroplasts.

An overnight culture of the yeast strain to be transformed was grown up in 5 mL YPDA to an OD_{600} of 3.0. A 200 µL aliquot was transferred to 200 mL YPDA which was incubated at 30°C and 140 rpm for 16 hours. The culture was centrifuged at 3,000 g for 5 minutes, washed once with dH_2O, and finally was resuspended in 35 mL 1.2 M sorbitol, 25 mM EDTA, 50 mM dithiothreitol, pH 8.0. The suspension was incubated at 30°C for 10 minutes with gentle shaking, centrifuged as before, and washed twice with 1.2 M sorbitol. The pellet was resuspended in 30 mL 1.2 M sorbitol, 10 mM EDTA, 0.1 M sodium citrate pH 5.8, and 0.3 mL of a 10 mg/mL Zymolyase-100T solution was added. The suspension was incubated at 30°C for 1 hour and then washed three times with 1.2 M sorbitol. The pellet was resuspended in 15 mL 1.2 M sorbitol, 10 mM $CaCl_2$, centrifuged and then resuspended in 1 mL of the same solution. 20 µL DNA (approximately 2 µg) was added to 200 µL cells and left at room temperature for 15 min. 1 mL 20% PEG-4000, 10 mM $CaCl_2$, 10 mM Tris.HCl pH 7.5 was added and left no longer than 10 minutes at room temperature. Cells were sedimented by centrifugation at 12,000 g for 5 seconds in a microfuge and resuspended in 100 µL 1.2 M sorbitol, 10 mM $CaCl_2$ plus 50 µL YPD. The tubes were incubated at 30°C for 20 minutes and diluted 10^3 fold in 1.2 M sorbitol and plated out in 6 mL regeneration agar onto selection agar plates. The plates were incubated 3 to 4 days at 30°C.

2.6.4. Lithium Acetate Transformation of *S. cerevisiae*

Three different methods, all derived form the method of Ito *et al.*, 1983, were used to transform *S. cerevisiae* with plasmid DNA, the transformation frequencies depending on the plasmid class used.

Conventional Lithium Acetate Method After Ito *et al.*, 1983

2 mL of a late log phase starter culture were used to inoculate 100 mL of pre-warmed YPDA. The initial cell density of the culture was determined with a Coulter

Counter, and from the initial density the approximate incubation time was estimated from a growth curve of the same strain under identical conditions to reach a final density of approximately 5.6 x 10^7 cells/mL. The cells were harvested by centrifugation at 15°C and 1,500 g for 5 minutes in two 30 mL aliquots. Each pellet was washed with 30 mL TE buffer pH 7.5, resuspended in 10 mL TE pH 7.5 and then 0.5 mL aliquots were transferred to test tubes. The tubes were incubated at 30°C and agitation (140 rpm) after the addition of 0.5 mL 0.2 M LiAc to each tube. 100 µL aliquots of the now competent yeast cells were mixed with no more than 15 µL plasmid DNA (approximately 10 µg DNA). It was important to include a positive and negative control at this step, i.e. a standard amount of plasmid DNA with known transformation frequency, and dH_2O as a negative control. All tubes were incubated at 30°C for 30 minutes without agitation. 110 µL 70% (w/v) PEG-4000 was added to each tube and then the tubes were incubated at 30°C for 60 minutes without agitation. The transformation mixtures were heat shocked at 42°C for exactly 5 minutes and then left at room temperature to cool down. The cells were sedimented by centrifugation for 60 seconds at 12,000 g in a microfuge, washed twice with 1 mL dH_2O each and were finally resuspended in 1 mL dH_2O each. 100 µL aliquots were spread out onto selective medium to screen for transformants (e.g. yeast minimal medium supplemented with amino acids except the one whose auxotrophic requirement was complemented by the transformed plasmid). The agar plates were incubated at 30°C for at least 4 days. Transformant colonies were streaked out onto minimal medium for further purification.

Simplified Lithium Acetate Method

2 mL of a late log - phase starter culture were used to inoculate 100 mL pre-warmed YPDA, the initial cell density of a 1 mL aliquot was determined with a Coulter Counter, and the culture was incubated at 30°C and 140 rpm, until an approximate cell concentration of 5.0 x 10^6 was reached as determined from a growth curve of the particular strain. Two 40 mL aliquots of the culture were centrifuged at 1,500 g and 5°C for 15 minutes. Each pellet was washed once with 10 mL dH_2O and then resuspended in 1 mL 0.1 M LiAc, 10 mM Tris.HCl pH 7.5, and incubated at 30°C for 60 minutes

with agitation (140 rpm). 200 µL of the suspension was added to 10 µg plasmid DNA in 15 µL TE buffer. A positive control with an amount of plasmid DNA of known transformation efficiency and a control omitting DNA were included at this step. The tubes were incubated at 30°C for 30 minutes and agitation (140 rpm). 1.2 mL 40% (w/v) PEG-4000 in 10 mM Tris.HCl pH 7.5, 0.1 M LiAc were added to each tube and then vortexed vigorously. The tubes were incubated at 30°C for 30 minutes with agitation (140 rpm), and subsequently heat shocked at 55°C for 1 minute and 37°C for 4 minutes The cells were centrifuged for 1 second at 12,000 g in a microfuge, washed twice with 1 mL TE and finally resuspended in 1 mL TE. 100 µL aliquots were spread out onto selective medium as described above and incubated at 30°C for at least 4 days. Transformant colonies were streaked out onto minimal medium and the presence of the correct plasmid was checked as described before.

Improved LiAc Method of Transformation (U. Certa, personal communication to M.D. Watson)

400 µL of a late log-phase starter culture was used to inoculate 25 mL pre-warmed YPDA. The initial cell density of a 1 mL aliquot was determined with a Coulter Counter, and the culture was incubated at 30°C and 140 rpm, until an approximate cell concentration of 5.0 x 10^7 was reached as determined from a growth curve of the particular strain. The culture was centrifuged at 10,000 g and 5°C for 5 minutes, and the pellet was washed once with 20 mL dH_2O. The cell pellet was finally resuspended in 20 mL 0.1 M LiAc and incubated at 30°C with agitation (140 rpm) for 60 minutes. The competent yeast cells were centrifuged at 4,000 rpm and room temperature for 10 minutes and then resuspended in 800 µL 0.1 M LiAc. 75 µL of the cell suspension was added to 10 µg plasmid DNA in 15 µL TE. A positive control with an amount of plasmid DNA of known transformation efficiency and control omitting DNA were included at this step. The tubes were incubated at 30°C for 30 minutes and agitation (140 rpm). The tubes were then vortexed gently, and 600 µL 40% (w/v) PEG-4000 in 10 mM Tris.HCl pH 7.5 was added. The tubes were incubated at 30°C for a further 60 minutes, and the cells were finally heat shocked at 42°C for 5 minutes. After

centrifugation at 12,000 g in a microfuge for 2 seconds, the cells were washed with 1 mL dH_2O once and were finally resuspended in 1 mL dH_2O. 100 µL aliquots were spread out onto selective medium as described above

2.6.5. Transformation of Yeast by Electroporation (Meilhoc *et al.*, 1990)

5 mL YPDA were inoculated with an average size colony of the yeast strain to be transformed. The culture was incubated at 30°C with agitation for 36 h. A 0.5 mL aliquot of this starter culture was inoculated into 100 mL pre-warmed YPDA and incubated at 30°C and 140 rpm until a cell density of 1 x 10^7 cells/mL was reached. The culture was centrifuged at 1,500 g and 4°C for 5 minutes and the pellets were resuspended in 1 mL 25 mM dithiothreitol, 10 mM Tris.HCl pH 8.0 in YPDA and statically incubated at 30°C for 10 minutes. The cells were washed twice with 1 mL 10 mM Tris.HCl pH 7.0, 270 mM sucrose, 1 mM $MgCl_2$ (electroporation buffer) by resuspending the pellets with gentle pipetting up and down in the buffer and spinning the cells down in a microfuge at 12,000 g for 15 seconds. Finally, the pellets were resuspended in 0.8 mL of the electroporation buffer (total volume with cells approximately 1 mL) and stored on ice until transformation. 48 µL of the cell suspension and 2 µL DNA solution (approximately 10 to 20 ng) were mixed in a microcentrifuge tube and transferred into a gene pulser cuvette (BioRad 0.2 cm electrode gap) and pulsed at 0.54 V (2.7 kV/cm) for about 15 to 20 ms (BioRad Gene Pulser). 1 mL YPDA was added to the transformation mix and it was incubated at 30°C for 1 hour to allow recovery and phenotypic expression of the selectable marker conferred by the plasmid (e.g. *URA3* with YCp50). Cells were harvested by centrifugation for 15 seconds at 12,000 g, resuspended in 100 µL dH_2O and directly plated out onto selective medium. The plates were incubated at 30°C for at least 4 days.

2.7. Protein Biochemistry Methods

2.7.1. Lowry Determination of Protein Concentrations (Lowry *et al.*, 1951)

100 µL of protein sample were added to 100 µL of a freshly prepared solution containing 0.5 mL 1% (w/v) $CuSO_4$, 0.5 mL 2.7% (w/v) sodium potassium tartrate, 5 mL 24% (w/v) Na_2CO_3, 0.66 mL 1 M NaOH and incubated at room temperature for 10 minutes. 500 µL of 1:19 diluted Folin-Ciocalteu reagent (Sigma) was added and the samples were incubated at 37°C for 30 minutes. The absorbance was read at 578 nm and the concentration of the sample was determined using known concentrations of BSA as standard.

2.7.2. Lämmli SDS Polyacrylamide Gel Electrophoresis (Lämmli, 1970)

SDS polyacrylamide gel electrophoresis (SDS PAGE) was performed using the Protean® II xi Slab Cell apparatus (Bio-Rad), as described in the manual.

SDS PAGE gels were prepared from an acrylamide/bis stock (30% T, 2.67% C) containing 292 g acrylamide and 8 g N'N'-bis-methylene-acrylamide in 1 L dH_2O which was not older than 30 days (see Table 2.6). Acrylamide/bis, dH_2O, and buffer were combined, degassed under vacuum for 5 minutes and the rest of the components added to the desired concentration. The gel apparatus was assembled for casting according to the manufacturers manual. After puoring, the gel was overlayed with dH_2O saturated butanol and was allowed to polymerize at room temperature for about 30 to 45 minutes. Butanol and unpolymerized acrylamide were poured off, the gel surface was washed with dH_2O and then the stacking gel was poured. The comb was inserted and the gel was allowed to polymerize for another 30 minutes. The comb was removed, the wells washed with dH_2O and the apparatus assembled for electrophoresis, filling both top and bottom reservoir with Tris-glycine buffer. Samples were loaded and electrophoresed at 30 mA/gel for 4 to 6 hour, using a Model 1000/500 power supply (Bio-Rad) at a constant current setting.

	Separating Gel			Stacking Gel
Monomer Concentration	12%	10%	7.5%	4.0%
Acrylamide/bis stock	40.0 mL	33.3 mL	25.0 mL	1.3 mL
dH_2O	33.5 mL	40.2 mL	48.5 mL	6.1 mL
1.5 M Tris.HCl pH 8.8	25.0 mL	25.0 mL	25.0 mL	
0.5 M Tris.HCl pH 6.8				2.5 mL
10% SDS	1.0 mL	1.0 mL	1.0 mL	100 µL
10% NH_4persulfate	500 µL	500 µL	500 µL	50 µL
TEMED	50 µL	50 µL	50 µL	10 µL

Table 2.6: Formulation for SDS separating and stacking gels.

2.7.3. Electrophoretic Blotting of Proteins

Electrophoretic blotting of proteins ("Western Blots") was done in a Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturers specifications.

The SDS PAGE gel to be blotted and a piece of nitrocellulose membrane of equal size were equilibrated in 100 mL ice-cold transfer buffer for 30 minutes. Two pieces of filter paper of equal size to the gel were also soaked in transfer buffer, the gel-holder was opened, and the gel/nitrocellulose sandwich could be assembled. One saturated filter was placed on the pre-wetted gray side of the assembly cassette, followed by the gel, the nitrocellulose membrane and the other filterpaper, avoiding the enclosure of air-bubbles. The cassette was closed and inserted such that the gray side faced the cathode. Transfer was performed at 100 V, 0.36 A for 4 hours with cooling on ice. After electrophoresis, the sandwich was disassembled, and the nitrocellulose membrane used in subsequent experiments. The gel could be stained with Coomassie blue to verify efficient tranfer.

The membrane was placed into a plastic box and approximately 20 mL blotting buffer was added, or until the blot was just covered. The membrane was incubated at

37°C for 75 minutes on a rotary shaker (50 rpm). It was then washed three times in blotting buffer for 10 minutes each at room temperature (50 rpm). 25 µL of the primary antibody in 20 mL blotting buffer was added to the box and incubated at 37°C for 135 minutes. The blot was washed three times in blotting buffer for 10 minutes at room temperature with gentle agitation. 20 µL secondary antibody alkaline phosphatase conjugate in 20 mL blotting buffer (1:1,000 dilution) was added to the membrane in the box and incubated at 37°C and 50 rpm for 2 hours. The blot was washed three times in blotting buffer for 10 minutes each at room temperature and 50 rpm and then rinsed once with 20 mL 1x Tris.HCl-salt pH 7.2. 333 µL 10 mg/mL NBT in 70% (v/v) dimethylformamide and 66 µL 25 mg/mL BCIP in 100% dimethylformamide was added to 10 mL alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris.HCl pH 9.5). The alkaline phosphatase substrate solution was added to the blot and developed at room temperature with gentle agitation until a positive control was obtained. The reaction was stopped by rinsing the filter in dH_2O, 1 M EDTA, and dH_2O successively. The filters were photographed, dried and stored in the dark. A deep purple to black band suggests the presence of a immunoreactive protein in the original gel.

2.7.4. Non-lysing Colony Immunoblot Assay

Qualitative secretion of soluble vacuolar proteins was tested with a dot-blot colony hybridization method (Rothman *et al.*, 1986).

Freshly grown colonies of the strains to be tested for secretion were picked from YPDA agar plates not older than 72 hours and patched onto YPDA agar plates together with a positive and negative control. A nitrocellulose filter was pre-wetted with 1x Tris.HCl-Salt (500 µL for a 8.5 cm filter) and placed onto the agar plate, the position of the filter was marked and the plate was incubated at 30°C for 8 hours, if secretion of PrA or PrB was tested, or for 14 hours if secretion of CPY was tested. After incubation, the filters were labeled and washed in a jet of dH_2O water. Each filter was placed into a petri-dish and processed as Western blots. The filters were photographed, dried and

stored in the dark. A deep purple to black patch over the position of a former colony signaled the secretion of the protein concerned.

2.7.5. Pulse-labelling and Immunoprecipitation of Yeast Proteins

Sphaeroplasts were prepared and labelled as described before (Robinson *et al.*, 1988), except that Zymolyase 100-T was used at 2 mg/mL instead of lyticase.

Cells were grown to mid-logarithmic phase in yeast minimal medium supplemented with 0.2% yeast extract. Four absorbance units at OD_{600} were harvested by centrifugation at 12,000 g for 20 seconds and resuspended in 1 mL 0.1 M Tris.SO_4 pH 9.4, 10 mM dithiothreitol and incubated at 30°C for 5 minutes. The cells were centrifuged again and resuspended in Wickerham's minimal medium adjusted to pH 7.4 and supplemented with 1.3 M sorbitol. Zymolyase was added to a final concentration of 1 mg/mL, and the cultures were incubated at 30°C for 45 minutes. Sphaeroplasts were pelleted by centrifugation at 5,000 g for 30 seconds, gently resuspended in 1 mL Wickerham's minimal medium supplemented with 1.3 M sorbitol and pulse-labelled by adding Tran[^{35}S]-label to 0.2 mCi/mL. The cells were incubated at 30°C for 20 minutes and chase was initiated by the addition of methionine to a final concentration of 50 µg/mL. After 30 minutes chase the culture was separated into sphaeroplast (intracellular) and supernatant (extracellular) fractions by centrifugation at 5,000 g for 5 minutes. The pellet fraction was washed once with Wickerham's medium containing 1.3 M sorbitol. Sphaeroplasts were broken by adding 0.5 mL NP40 lysis buffer (1% (v/v) NP40, 0.5% (w/v) DOC, 0.1% (w/v) SDS, 10 mM Tris.HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.4 M galactose and 40 µg/mL PMSF). Tubes were left on ice for 30 minutes, 0.3 g acid-washed glass beads were added and then vortexed for 30 seconds. Debris was sedimented by centrifugation at 10,000 g for 10 minutes. Both intracellular and extracellular fractions were mixed with an equal volume of 1% (v/v) NP40, 10 mM Tris.HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.4 M galactose and 40 µg/mL PMSF, then 50 µL protein-A-sepharose slurry (1:1 in NP40 buffer) was added to each tube and left on ice for 30 minutes. After centrifugation at 10,000 g for 5 minutes, 2 µL of

affinity purified rabbit antiserum against the appropriate vacuolar enzyme was added to the supernatant, followed by incubation for 1 hour at 4°C. 50 µL protein-A-sepharose slurry (see above) was added and the mixture was incubated on ice for an additional 30 minutes with occasional shaking. Beads were collected by centrifugation for 15 seconds and washed three times with 1 mL 0.2% NP40 buffer, twice with 0.2% NP40 buffer containing 0.5 M NaCl and once with 1.0 mL 10 mM Tris.HCl pH 7.5. The washed protein-A-sepharose antibody-antigen conjugates were resuspended in 50 µL SDS sample buffer (200 mM Tris.HCl pH 8.8, 0.5 M sucrose, 5 mM EDTA, 0.01% bromophenol blue, 1% (w/v) methionine, 4% (w/v) SDS, 10 mM dithiothreitol), boiled for 3 minutes and separated on a 7.5% acrylamide gel. Fluorography using PPO has been described elsewhere (Laskey and Mills, 1975). Pre-flashed Fuji-XR film was exposed to the dried gels at -84°C for 3 days for densitometric examination, and for 5 to 7 days for photographic presentation.

2.7.6. Immunoprecipitation of CPY in Intra- and Extracellular Yeast Fractions

A simplified protocol could be used to immunoprecipitate CPY from culture supernatant and broken yeast sphaeroplasts, since the anti-CPY antibody used in this study was still able to bind to CPY under denaturing conditions where most other proteins could not bind non-specifically to the immuno-sorb matrix used (C.K. Raymond, unpublished observations).

Cells were grown to an OD_{600} 1.0 and the equivalent of one absorbance unit was harvested and resuspended in 1.0 mL yeast minimal medium, 50 mM KPO_4 pH 5.7, 2 mg/mL BSA, and incubated at 30°C for 10 minutes. 500 µL was transferred to a screw-cap microfuge tube and Tran[^{35}S]-label was added to a concentration of 100 µCi/tube. The tubes were incubated for 10 minutes at 30°C and then chased by adding 50 µL methionine/cysteine (5 mg/mL each) and incubation for another 30 minutes. The pulse-chase label reaction was stopped by adding 5 µL 1 M NaN_3. Cells were harvested and the supernatant was transferred to new tubes containing 10 µL 100x proteinase inhibitor cocktail (0.5 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin A) each. This tube

represents the external fraction. 150 µL freshly prepared sphaeroplast mix (1.4 M sorbitol, 50 mM Tris.HCl pH 7.4, 2 mM $MgCl_2$, 10 mM NaN_3, 3 µL/mL β-mercaptoethanol, 15 µg/mL zymolyase 100-T, 25 µL/mL glusulase) was added to the cell pellet (internal fraction) and incubated at 30°C for 30 to 45 minutes. 100 µL 10x IP buffer (0.9 M Tris.HCl pH 8.0, 1% SDS, 1% Triton X-100, 20 mM EDTA) and 150 µL dH_2O was added to the external fraction and heated to 100°C for 5 minutes. The internal fraction was supplemented with 10 µL 100x proteinase inhibitor cocktail, 100 µL 10x IP buffer and 519 µL dH_2O and also incubated at 100°C for 5 minutes. After cooling to room temperature, samples were preadsorped by adding 50 µL IgSorb and 221 µL dH_2O to both samples and incubating them on ice for 15 minutes. The tubes were centrifuged for 5 minutes at 12,000 g and 950 µL supernatant was transferred to a new tube containing 10 µL anti-CPY antiserum diluted 1:10 in 1x immunobuffer (10 mM Tris.HCl pH 8.0, 0.1% SDS, 0.1% Triton X-100, 2 mM EDTA). After 1 hour incubation on ice with occasional mixing 50 µL IgSorb was added and then incubated for another hour on ice. The CPY/anti-CPY/IgSorb complex was sedimented at 12,000 g for 1 minute and pellets were washed twice in 1 mL immunobuffer. Washed pellets were resuspended in 40 µL 2x SDS loading buffer and then electrophoresed as described above (2.7.2.).

2.7.7. Expression and Isolation of Recombinant Proteins from *E. coli*

The expression of recombinant proteins in E. coli was facilitated by cloning the open reading frame of interest into the expression vector series pEXP1-3. These vectors are designed to subclone blunt ended or cohesive termini fragments into the Sma I, Xba I, Sal I or Hind III sites of the multiple cloning site which are linked to the P_{lac} P_{tac} promoters (Muchmore *et al.*, 1989).

The open reading frame of the protein of interest was cloned into the pEXP expression vector in frame with the N-terminus of T4 lysozyme encoded by the vector. Constructs were transformed into competent MC1061 and tested for expression by diluting an overnight culture of MC1061 harbouring the desired plasmid 100 fold into

fresh Luria broth containing ampicillin. The culture was grown at 37°C for 1 hour, IPTG added to 1-2 mM and incubated at 37°C for 3 hours to induce recombinant protein expression. A control, omitting IPTG, was included at that point to allow a comparison. 1.5 mL cells were harvested by centrifugation at 12,000 g for 1 minute, 150 µL protein sample buffer was added and samples were incubated at 100°C for 2 minutes. The contents of the tubes were vortexed, centrifuged at 12,000 g for 5 minutes and then 5 µL of the sample was loaded onto a SDS PAGE gel and electrophoresed under standard conditions. The recombinant protein is normally well expressed and detectable on the gel in the inuced sample. Occasionally, as was the case for recombinant CPY, the recombinant protein was of identical size to abundant bacterial proteins and could not be detected by the rapid screening protocol described above. In this case, a small scale extraction of the recombinant protein was performed.

50 mL Luria broth was inoculated with 0.5 mL of an overnight culture of the desired clone and then induced with IPTG to 2 mM after 1 hour as described above. A non-induced control was included at this point. The cultures were incubated at 37°C for 3 hours and then harvested by centrifugation at 3,000 g for 5 minutes. The pellets were frozen at -84°C, thawed and resuspended in 1 mL STEE (50 mM sucrose, 25 mM Tris.HCl pH 8.0, 5 mM EDTA, 5 mM EGTA) supplemented with 10 mg/mL lysozyme. This lysis mix was incubated 10 - 15 minutes at room temperature with occasional mixing. 40 mL distilled water was added and shaken at 200 rpm for 2 minutes. 1 mL 1 M Tris.HCl pH 7.5, 1.6 mL 100mM $MgCl_2$, 40 µL 1M PMSF, 200 µL of RNAse/DNAse (10 mg/mL RNAse, 1 mg/mL DNAse in 50% (v/v) glycerol, 10 mM Tris.HCl pH 7.5) was added and then agitated 10 - 15 minutes at room temperature. After centrifugation at 15,000 g for 10 minutes, the supernatant was saved and 2 mL of it was precipitated by adding 8 mL of acetone, mixing, chilling at -20°C for 2 hours, pelleting the precipitate at 15,000 g, removing the supernatant and resuspending the pellet in 200 µL protein sample buffer. The pellet from above was resuspended in 20 mL of 50 mM Tris.HCl pH 7.5, 4 mM MgCl2, 1% (v/v) Triton X-100, 0.1 mL RNAse/DNAse solution (see above) and agitated 10 - 15 minutes. 20 mL 50 mM Tris.HCl pH 7.5, 20 mM EDTA, 1% (v/v) Triton X-100 was added and then shaken for

an additional 10 - 15 minutes. Samples were centrifuged as before. 2 mL of the supernatant was kept and acetone precipitated as before. The pellet fraction, which should contain the foreign protein, was resuspended in 0.5 mL of protein sample buffer. An analytical SDS PAGE of the induced whole cell extracts, the first and second supernatant fractions, and the Triton X-100 washed pellet fraction was run.

If the recombinant protein was to be expressed on a large scale, then usually a 250 mL culture was induced and the above extraction procedure scaled up proportionally. If the analytical SDS PAGE revealed the presence of the recombinant protein, the corresponding fraction (always the Triton X-100 washed pellet) was taken and loaded onto a preparative SDS PAGE (15 x 15 x 0.5 cm). Electrophoresis was performed at 50 mA constant current. The gel was stained with 250 mM KCl, 1 mM dithiothreitol at 4°C for several hours, or until bands were visible. It was often helpful to cut a slice from the preparative gel and stain it with Coomassie Blue in order to unambiguously identify the correct protein band. The band was excised with a razor blade and destained in distilled water for 1 hour. The slice was homogenized with a blender in 5 mL SDS elution buffer (2% (w/v) SDS, 50 mM Tris.HCl pH 6.8, 100 mM NaCl, 2mM EDTA, 1 mM dithiothreitol), and the slurry was transferred to a falcon tube. The homogenizer was rinsed with an additional 5 mL of the SDS elution buffer and the samples were pooled. Samples were shaken at 37°C for 4 hours. An additional 10 mL of the elution buffer was added to the slurry and extracted as before. Supernatant fractions were pooled and precipitated with 80 mL acetone at -20°C for 4 hours. The precipitate was centrifuged at -20°C and 15,000 g for 20 minutes and pellets dried and resuspended in 1 mL 1% (w/v) SDS. 1 µL of the protein sample was run on an analytical SDS PAGE, against a known standard (BSA) to assess yield and purity.

2.7.8. Production of Polyclonal Antisera in Rabbits

Usually two female New Zealand white rabbits, which were maintained on a yeast extract free RABMA rabbit maintenace diet (Special Diets Services), were immunized with one antigen to yield a polyclonal antibody response.

200 µg antigen in 1% (w/v) SDS, 2.7 mL Freunds complete (Sigma), 70 µL 20x PBS, dH_2O to 4 mL were emulsified by repeated voxtexing for at least 10 minutes before injection, and each rabbit was injected intradermally at six locations on the back with 330 µL of the emulsion. After 28 days, the first booster injection was given. 100 µg antigen in 1% (w/v) SDS, 2.7 mL Freund incomplete (Sigma), 70 µL 20x PBS, dH_2O to 4 mL were emulsified, and 330 µL aliquots were injected as before at six locations per rabbit. After 12 days, the rabbits were test-bled, the blood was coagulated at 37°C for 30 minutes, and the serum supernatant was stored at 4°C. Aliquots were taken to determine Ab-titer and specificity in an ELISA and Western blot, respectively. After 28 days, the second booster was given by injecting 330 µL aliquots of 50 µg antigen in 1% (w/v) SDS, 70 µL 20x PBS, dH_2O to 4 mL as before. After 10 days, the rabbits were test bled, and if the antibody titer was sufficiently high as determined in an ELISA, the rabbits were terminated by cardiac puncture. Blood was allowed to clot at 37°C, red blood cells were pelleted by centrifugation at 3,000 g for 10 minutes, and the serum supernatant was supplemented with NaN_3 to 10 mM and stored at -84°C in 5 mL aliquots.

2.7.9. Adsorption of Antibodies

Polyclonal antibodies raised by injection of rabbits with purified yeast proteins were purified by adsorption with yeast cells deleted for the corresponding gene to bind all non-specific antibodies.

5 mL YPDA were inoculated with a fresh colony of the yeast strain and were incubated at 30°C for 6 hours with agitation. Four 1 mL aliquots were centrifuged at 12,000 g in pre-weighted microcentrifuge tubes for 5 minutes and were washed with 1 mL Tween-20 precoat buffer each. After thorough draining the weight of the pellets was determined. 30 µL of the antibody to be purified was added per 1 mg pellet, mixed well and the tube left at room temperature for 30 minutes. Cells were pelleted by centrifugation at 12,000 g for 5 minutes and the supernatant transferred to another tube containing the yeast cells and the binding step was repeated. Finally, the supernatant

was taken off the pellet, 1 M NaN_3 was added to a final concentration of 10 mM, the adsorbed antibody was dispersed in 25 µL aliquots and stored at -20°C.

2.7.10. Enzyme-linked Immunoadsorbent Assay (ELISA)

A 96 well cluster plate with flat bottom was coated with antigen by filling each well with 80 µL 2 µg/mL of the antigen in 50 mM carbonate buffer pH 9.2, as well as another cluster with 2 µg/mL BSA as non-specific antigen control. The clusters were incubated overnight in the dark at room temperature in a humid chamber. They were then washed 5 times with Tris.HCl-Salt pH 7.2, 200 µL blocking buffer was added to each well and the clusters incubated at 37°C for 1 hour. The wells were washed 5 times with Tris.HCl-Salt buffer, and 100 µL of a decimal dilution series of the antibody to be titrated was given into each well of both plates, leaving some wells with buffer only as controls. After incubation at 37°C for 90 minutes the wells were washed 5 times as before, and 100 µL secondary antibody horseraddish peroxidase conjugate in a 1:1,000 dilution in blocking buffer was added to each well. The cluster then was incubated at 37°C for 90 minutes and then washed five times as before. 50 µL of a peroxidase assay mix (1 mg/mL p-nitrophenoxyzol, 5 µL/mL 30% (v/v) H_2O_2) were added to each well, and the absorbance was read at 495 nm immediately, after 10, and after 30 minutes. Results were corrected, taking account of controls and non-specific antigen binding.

2.8. Enzyme Assays

2.8.1. Invertase Assay (Johnson *et al.*, 1987)

Yeast strains were grown in 3 mL MV-Pro-Fructose medium at 30°C for 72 hours. 100 µL of the initial culture was added to 3 mL MV-Pro-Fructose medium and incubated at 30°C for 36 hours. After centrifugation for 10 minutes at 3,000 g, pellets were washed with 3 mL 10 mM NaN_3, and resuspended in 1.5 mL 0.1 M sodium acetate pH 5.5. A 0.5 mL aliquot of each sample was taken and used for the invertase

assay directly (periplasmic fraction). 50 µL 10% (v/v) Triton X-100 was added to another 0.5 mL sample, which then was frozen in liquid nitrogen and subsequently thawed and assayed for invertase (periplasmic and intracellular fraction). 25 µL of the samples were given to 50 µL sodium actate pH 4.9 in a test tube, and 25 µL 0.5 M sucrose were added to start the reaction. Tubes were incubated at 30°C for 10 minutes and then placed into a boiling water bath for 3 minutes to stop the reaction after the addition of 100 µL 0.5 M KPO_4 pH 7.0. Samples were cooled down to 30°C, and 1 mL of a freshly prepared solution containing 1 mL 84 µg/mL glucose oxidase, 10 µg/mL horseradish peroxidase, 0.9 mL/mL 0.1 M KPO_4 pH 7.0, 0.5 mL 0.3 mg/mL o-dianisidine, and 8.5 mL 45% (v/v) glycerol was added to each tube. These were incubated at 30°C for another 20 minutes. 0, 20, 40 and 60 µM/L glucose standards were included. 1.5 mL 6 M HCl was added to stop the reaction and the developed red colour was read at 450 nm in a spectrophotometer. This step measured the glucose released during the enzymatic hydrolysis of sucrose in the first step. A 100 µL sample of the cell suspension was taken for an estimate of the cell number. Another 100 µL sample of the suspension was used for a protein estimation according to the method of Lowry. The secreted (periplasmic) and total invertase activity was related to the total protein concentration and cell number, and the relative degree of invertase secretion could be determined.

2.8.2. α-glucosidase Assay

Cells to be analyzed for α-glucosidase secretion were grown in 3 mL yeast minimal medium supplemented with all essential amino acid for 36 hours at 30°C. Cells were harvested by centrifugation at 3,000 g for 5 minutes, pellets were resuspended in 1.5 mL 0.1 M $NaPO_4$ pH 7.0 and subdivided into two 0.5 mL fractions for the enzyme assay. The rest of the suspension was kept for protein estimations and cell counts. To determine the total fraction of α-glucosidase, 50 µL 10% (v/v) Triton X-100 was added to one aliquot of cells, followed by the addition of half the volume of acid washed glass beads and vigorous vortexing for 2 minutes. The debris was sedimented and the

supernatant kept for assaying the total α-glucosidase activity. The secreted (periplasmic) fraction of α-glucosidase was determined from the other, non Triton X-100 treated corresponding cell suspension. 25 µL of the crude cell extract was added to 500 µL of a solution containing 40 mM p-nitrophenyl-α-D-glucopyranoside, 40 mM sodium acetate pH 6.5. A control containing buffer only instead of the cell extract was included at that step. Tubes were incubated at room temperature for 15 minutes, and the reaction was terminated by the addition of 200 µL 10% (w/v) TCA. They were centrifuged for 10 minutes at 12,000 g in a microfuge and the supernatant mixed with 0.5 mL 1 M glycine pH 10.4 and the absorbance measuerd at 400 nm. Assuming an extinction coefficient of 1.9×10^4, the activity was expressed in µmol p-nitrophenyl librated per minute.

2.8.3. α-mannosidase Assay (Opheim, 1978)

Cells to be analyzed for α-mannosidase secretion were grown in 3 mL yeast minimal medium supplemented with all essential amino acid for 36 hours at 30°C. Cells were harvested by centrifugation at 3,000 g for 5 minutes, pellets were resuspended in 1.5 mL 0.1 M $NaPO_4$ pH 7.0 and subdivided into two 0.5 mL fractions for the enzyme assay. The rest of the suspension was kept for protein estimations and cell counts. To determine the total fraction of α-mannosidase, 50 µL 10% (v/v) Triton X-100 was added to one aliquot of cells, followed by the addition of half the volume of acid washed glass beads and vigorous vortexing for 2 minutes. The debris was sedimented and the supernatant kept for assaying the total α-mannosidase activity. The secreted (periplasmic) fraction of α-mannosidase was determined from the other, non Triton X-100 treated corresponding cell suspension. 25 µL of the crude cell extract was added to 500 µL of a solution containing 40 mM p-nitrophenyl-α-D-mannopyranoside, 40 mM sodium acetate pH 6.5. A control containing buffer only instead of the cell extract was included at that step. Tubes were incubated at room temperature for 15 minutes, and the reaction was terminated by the addition of 200 µL 10% (w/v) TCA. They were centrifuged for 10 minutes at 12,000 g in a microfuge and the supernatant mixed with

0.5 mL 1 M glycine pH 10.4 and the absorbance measuerd at 400 nm. Assuming an extinction coefficient of 1.9 x 10^4, the activity was expressed in µmol p-nitrophenyl librated per minute.

2.9. Light Microscopy

2.9.1. Normarski Contrast

Starter cultures of the yeast strain to be examined were set up by inoculating 5 mL YPDA with a colony and incubation at 30°C with agitation for 48 hours. 100 µL of the stationary phase cells were used to inoculate 3 mL YPDA. The cultures were incubated at 24°C for 24 hours at 200 rpm. 5 µL aliquots of each culture were transferred onto microscope slides, covered with a cover-slip and observed under 1,000x Normarski contrast optics with a Nikon inverted microscope. A black and white photograph of a typical population of each strain was taken with an attached Olympus OM2 camera onto T_{max} film (400 ASA).

2.9.2. Fluorescence Microscopy

Labeling of the mutants with FITC and quinacrine was performed as described in Banta *et al.*, 1988.

5 mL YPDA were inoculated with a colony and incubatied at 30°C with agitation for 48 hours. 100 µL of the stationary phase cells were used to inoculate 3 mL YPDA. The cultures were incubated at 24°C for 24 hours at 140 rpm. 1 mL of the cell suspension was centrifuged at 5,000 g for 20 seconds and resuspended in 1 mL YPDA supplemented with 50 mM sodium citrate pH 5.5 and 10 µg/mL FITC in DMSO. After 10 minutes incubation at 25°C with agitation, cells were centrifuged as above, washed once with YPDA and resuspended in 100 µL 100 mM KPO_4 pH 7.5, 2% glucose. Cells were mounted onto polylysine coated microscope slides and viewed under a fluorescence microscope. For quinacrine vital stainig of vacuoles, cells were grown as

above, and quinacrine was added to the culture to 175 μM in YPDA pH 7.6. After a 5 minute incubation period at 30°C, cells were centrifuged as above and mounted onto slides without washing. Specimens were observed under fluorescence microscopy using a blue-filter.

2.9.3. Indirect Immunofluorescenece Microscopy

Yeast cells were prepared for indirect immunofluorescence microscopy using a modified protocol to the one described in Roberts *et al.*, 1991.

Strains to be examined were streaked out to near confluence on fresh YPD agar plates, unless plasmid selection needed to be maintained. After overnight incubation at 30°C, colonies were taken off the surface of the agar plate, resuspended to an OD_{600} of about 0.25 in 10 mL of YPD and agitated at 30°C for 2-4 hours. The cultures were pre-fixed by the addition of 1.2 mL of 37% (v/v) formaldehyde and agitation at 30°C for 60 minutes. Fixative was freshly prepared by adding 2 g paraformaldehyde to 50 mL dH_2O containing 375 μL 6M NaOH and heating until the paraformaldehyde dissolves. The solution was neutralized with 0.68 g KH_2PO_4 and a pH of about 6.5 confirmed. Cells were centrifuged in a clinical centrifuge for 20 seconds and the supernatant was aspirated away. Pellets were resuspended in 2 mL fixative and shaken at room temperature to 30°C 12 to 18 hours. Cells were harvested in a clinical centrifuge, resuspended in 1 mL 200 mM Tris.HCl pH 8.0, 20 mM EDTA, 1% (v/v) β-mercaptoethanol and were transfered to microcentrifuge tubes. After 10 minutes cells were sedimented by low speed centrifugation for 20 seconds and resuspended in freshly prepared sphaeroplast solution (1.2 M sorbitol, 50 mM KPO_4 pH 7.3, 1 mM $MgCl_2$, 15 μg/mL zymolyase 100-T, 25 μL/mL glusulase). After 35 minutes incubation with gentle shaking, sphaeroplasts were pelleted at slow speed and washed once with 1.2 M sorbitol. Pellets were resuspended in 500 μL 1.2 M sorbitol and treated with SDS. SDS denaturing conditions depended strongly on the primary antibody used, and are shown in Table 2.7.

Primary antibody	[SDS] added	Incubation period
a-Pho8p	500 µL 10% (w/v) SDS	5 minutes
a-60kD	125 µL 2% (w/v) SDS	1 minute

Table 2.7: Denaturing conditions for cells prepared for indirect immunofluorescence with specific primary antibodies.

After denaturation, cells were washed twice in 1 mL 1.2 M sorbitol, and were finally resuspended in 1 mL 1.2 sorbitol. 40 µL of cell suspension was added to each well of a polylysine coated microscope slide and allowed to settle for 15 minutes at room temperature in a moist chamber. The fluid was aspirated off and the slide washed twice with 20 µL PBS-BSA (1x PBS, 5 mg/mL BSA, 10 mM NaN_3). 20 µL PBS-BSA was added and the slides were left in a humid chamber for 15 minutes. The PBS-BSA was aspirated off and 10 µL of the primary antibody solution added. For polyclonal antisera a 1:10 working solution was used; monoclonal antibodies were used in undiluted form. After 1 hour incubation in a humid chamber the wells were washed six times with PBS-BSA and the second antibody was added. In most cases, amplification of the flourescence signal was required. For amplification, 10 µL of a 1:1,000 dilution of goat anti-rabbit IgG or goat anti-mouse IgG were added and incubated for 1 hour. After six washes a 1:1,000 dilution of the third antibody (rabbit anti-goat IgG or mouse anti-goat IgG) was added and left for another hour. After six washes a 1:200 dilution of final antibody (FITC-goat anti-rabbit IgG or FITC-goat anti-mouse IgG) was added and the slides were incubated for 1 hour as before. After nine washes, 20 µL of mounting medium (Citifluor containing 0.1 µg/mL DAPI) per well was added and slides were covered with 24 x 60 mm coverslips. After 5 minutes two edges were sealed with fingernail polish to fix the coverslip into position. Slides were ready for viewing and can be stored successfully for 1 to 2 months at -20°C.

Slides were examined under an Axioplan fluorescence photomicroscope (Carl Zeiss Inc, Thornwood, NY) fitted with filters for DAPI and FITC fluorescence. Each

specimen was photographed under Normarski contrast (auto-setting), DAPI (2 seconds) and FITC (4, 8, and 15 seconds) fluorescence onto T_{max} 400 film.

2.10. Electron Microscopy

Yeast cells were prepared for electron microscopy using a modified procedure to the one described in Banta *et al.*, 1988.

10 mL yeast minimal medium supplemented with all essential amino acids were inoculated with 200 µL of a stationary phase starter culture and grown at 24°C for 5 hours on a rotary shaker (200 rpm), until an OD_{600} of 0.3 was reached. The cells were separated from the minimal medium by centrifugation (12 minutes at 1,500 g and room temperature), resuspended in 10 mL YPDA and allowed to grow for 1.5 hours under the same conditions as described above. The cells were harvested by centrifugation (see above), resuspended in 1 mL dH_2O, transferred to microcentrifuge tubes and centrifuged at 12,000 g for 2 minutes. The pellets were used in subsequent protocols to enhance different subcellular compartments.

To assess the effect of high temperature on temperature sensitive strains, these strains were incubated at 37°C for 1.5 hours after shifting to YPDA medium. Furthermore, the first fixation step was carried out at 37°C rather than room temperature as for temperature resistant strains.

2.10.1. Enhancement of Yeast Vacuoles and Double Membranes

A ferrocyanide-reduced osmium - thiocarbohydrazide - ferrocyanide-reduced osmium fixation method (Willingham *et al.*, 1984) was employed to enhance yeast vacuoles and double-membrane structures.

Cells prepared as above (2.10.) were fixed for 2 hours at room temperature in 0.5 mL 3% (v/v) glutaraldehyde, 0.1 M sodiumcacodylate pH 6.8, 5 mM $CaCl_2$. The fixed cells were centrifuged at 12,000 g in a microfuge for 20 seconds, washed in 0.5 mL 100 mM Tris.HCl pH 8.0, 25 mM dithiothreitol, 5 mM EDTA, 1.2 M sorbitol (TDES), and finally were resuspended in 0.5 mL TDES and incubated for 10 minutes at 30°C. The

cells were centrifuged, washed once with 0.1 M KPO_4 (pH 5.8 with solid citric acid), 1.2 M sorbitol, and resuspended in the same buffer containing 4 mg/mL Zymolyase 100-T. Sphaeroplasts were produced by incubation at 37°C for 45 minutes. The sphaeroplasts were centrifuged at 1,500 g for 30 seconds, washed once with 1 mL 0.1 M sodium cacodylate pH 6.8, 5 mM $CaCl_2$ (cacodylate buffer), and finally were resuspended in 200 µL 1% (w/v) OsO_4, 1% (w/v) K-ferrocyanide in cacodylate buffer and incubated 30 minutes at room temperature. After four washes in 1 mL dH_2O, the sphaeroplasts were resuspended in 1 mL 1% (w/v) thiocarbohydrazide (freshly made up at 58°C, followed by sterile filtration through a 0.45 µm millipore filter after cooling down to room temperature immediately before use) and left at room temperature for 5 minutes. The samples were washed four times with 1 mL dH_2O and stained with 1% (w/v) OsO_4, 1% (w/v) K-ferrocyanide in cacodylate buffer for 3 minutes at room temperature. After four washes with dH_2O, the fixed and stained sphaeroplasts were dehydrated through an ethanol series (12.5% (v/v), 12.5% (v/v), 25% (v/v), 25% (v/v), 50% (v/v), 50% (v/v), 75% (v/v), 75% (v/v), 99% (v/v), 99% (v/v), dry 100%). The ethanol was changed after at least 30 minutes between each step by centrifugation for 30 seconds at 12,000 g, resuspended, and finally left overnight in dry 100% ethanol. The samples were resuspended in 1 mL Spurr:dry ethanol (1:1) and were left for 8 hours at room temperature. Cells were sedimented by centrifugation at 12,000 g for 45 seconds, and were resuspended in Spurr resin. The resin was discarded after 24 hours, and fresh Spurr was added. The in Spurr resuspended sphaeroplasts were transferred to a 24 block casting mold and left to polymerize for 15 to 17 hours at 70°C. Ultra-thinsections were cut on a Sorvall Ultramicrotome MT-2 with a freshly broken glass knife, mounted onto 200-mesh copper grids (no coating), and dried on filter-paper overnight. The sections were stained with lead citrate (Reynolds *et al.*, 1963) for 45 seconds in a CO_2 free atmosphere, destained in 0.02 M NaOH for 2 seconds, and washed on a droplet of dH_2O for 5 seconds. The sections were examined with a Philips TEM400 electron microscope after they were sufficiently dry.

2.10.2. Fixation after Byers and Goetsch, 1975

Cells prepared as above (2.10.) were resuspended in 1 mL 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate pH 6.8, 5 mM $CaCl_2$ at 20°C for 30 minutes, and at 0°C for 16 hours or overnight. The fixed cells were centrifuged at 12,000 g in a microfuge for 20 seconds, washed in 0.5 mL 100 mM Tris.HCl pH 8.0, 25 mM dithiothreitol, 5 mM EDTA, 1.2 M sorbitol (TDES), and finally were resuspended in 0.5 mL TDES and incubated 10 minutes at 30°C. The cells were centrifuged, washed once with 0.1 M KPO_4 (pH 5.8 with solid citric acid), 1.2 M sorbitol, and resuspended in the same buffer containing 4 mg/mL Zymolyase 100-T. Sphaeroplasts were produced by incubation at 37°C for 45 minutes. The sphaeroplasts were centrifuged at low speed for 30 seconds, and incubated in 2% (w/v) osmium tetroxide in 0.1 M sodium cacodylate pH 6.8, 5 mM $CaCl_2$ for 1 hour at 0°C. Post-fixed sphaeroplasts were washed once with dH_2O, resuspended in 1 mL 2% (w/v) uranyl acetate, dehydrated in an ethanol series as above, and were embedded in Spurr resin as described before. Ultra-thinsections were cut on a Sorvall Ultramicrotome MT-2 with a freshly broken glass knife, mounted onto 200-mesh copper grids (no coating), and dried on filter-paper overnight. The sections were stained with 2% (w/v) uranyl acetate for 45 seconds and lead citrate (Reynolds *et al.*, 1963) for 45 seconds in a CO_2 free atmosphere, and were dried on filter paper overnight and the sections were examined with a Philips TEM400 electron microscope after they were sufficiently dry.

CHAPTER 3

RAISING POLYCLONAL ANTISERA AGAINST RECOMBINANT CARBOXYPEPTIDASE Y

3.1. Introduction

The yeast vacuole is considered to represent the major digestive compartment of the cell (Wiemken *et al.*, 1979) and also to play an important role in the storage of metabolites and amino acids (Klionsky *et al.*, 1990). Most of its soluble and membrane associated protein constitutents transit through the secretory path (reviewed in Klionsky *et al.*, 1990), although some proteins are imported directly, such as fructose 1,6-bisphosphatase (Chiang and Schekman, 1991) and α-mannosidase (Yoshihisa and Anraku, 1990). The soluble vacuolar proteases have been characterized extensively in terms of cellular function and biosynthesis (Jones, 1991; Klionsky *et al.*, 1990; Wolf, 1986). A soluble vacuolar protein in the yeast *S. cerevisiae* which has received much attention and of which a great deal of information concerning transport, targeting and biological function is known, is carboxypeptidase Y (CPY).

CPY is a serine protease involved in a series of proteolytic events in the vacuole (Achstetter and Wolf, 1985). The structural gene for CPY, *PRC1*, has been cloned (Stevens *et al.*, 1986) and sequenced (Valls *et al.*, 1987). It encodes a 58 kDa precursor protein, pre-pro-CPY (ppCPY), that is imported into the endoplasmic reticulum (Stevens *et al.*, 1982). The pre-pro-protein undergoes signal peptidase dependent proteolytic processing (Stevens *et al.*, 1982) and is core glycosylated in the ER (Ballou *et al.*, 1990). The signal peptide is cleaved between Ala-20 and Ile-21 so the first aminoacid of pro-CPY (p1CPY) is amino acid 21 of the predicted amino acid sequence (Blachly-Dyson and Stevens, 1987). The core glycosylated p1CPY, which migrates at 67 kDa on a denaturing SDS-PAGE (Klionsky *et al.*, 1990), exits the ER and proceeds to the Golgi apparatus (Stevens *et al.*, 1982). The core-glycosylated p1CPY undergoes

further carbohydrate modification in transit through the Golgi (Ballou *et al.*, 1990; Stevens *et al.*, 1982) such that the CPY precursor now has a molecular weight of approximately 69 kDa (p2CPY) (Klionsky *et al.*, 1990). It is thought that at some stage in the Golgi apparatus sorting to the vacuole occurs (Klionsky *et al.*, 1990; Stevens *et al.*, 1982). The signal responsible for correct targeting of pro-CPY to the vacuole is located within the first 30 amino acids of the N-terminus of the pro-peptide (Valls *et al.*, 1987), and is defined by the four successive amino acid residues QRPL (amino acids 24 to 27 of the predicted amino acid sequence of *PRC1*) (Valls *et al.*, 1990). On delivery to the vacuole, proteinase A (PrA) and proteinase B (PrB) dependent proteolytic processing of p2CPY to the mature 61 kDa form occurs (Ammerer *et al.*, 1986; Jones, 1991; Stevens *et al.*, 1982). PrA was thought to be the major processing enzyme in the maturation of CPY (Ammerer *et al.*, 1986). However, the site-specificity of PrA, which preferentially cleaves peptide bonds between large hydrophobic amino acids (Dreyer, 1989), does make the established cleavage of the CPY pro-peptide between Asn-111 and Lys-112 by PrA alone unlikely. The current view on the vacuole dependent maturation of p2CPY to CPY suggests that both PrA and PrB are equally involved in the maturation and activation of p2CPY (Mechler *et al.*, 1987). PrA will remove most of the CPY pro-sequence, and subsequently PrB will trim off the residual aminoacids of the propeptide (Jones, 1991). The CPY processing and transit pathway has been reconstituted *in vitro* (Vida *et al.*, 1990). Although recent evidence suggests that different vacuolar proteases are sorted by different mechanisms (Klionsky *et al.*, 1988; Paravicini *et al.*, 1992; Raymond *et al.*, 1992), the sorting pathway of CPY is well established and some mutants that missort vacuolar enzymes were isolated on the basis of secretion of CPY (Rothman *et al.*, 1986) or secretion of a ppCPY-invertase fusion (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988) which has the CPY signal peptide and the vacuolar sorting signal fused to the secretory enzyme invertase. The mislocalization of CPY into the periplasm has been attributed to defects in vacuolar protein sorting (Rothman *et al.*, 1989a) and vacuole biogenesis (Banta *et al.*, 1988). As described above, CPY is differentially modified in different compartments of the secretory pathway and upon delivery to the vacuole. The different forms of CPY can easily be

distinguished by their associated size difference on SDS-PAGE. In addition all forms of CPY as well as the secreted form of the enzyme can readily be retrieved by immunoprecipitation. These considerations led to the choice of CPY as a reporter for protein sorting and vacuole biogenesis defects in screening mutants in this study, as well as in a number of previous studies (for review see Klionsky *et al.*, 1990; Raymond *et al.*, 1992).

The purification of yeast proteins for raising antibodies is often difficult because the presence of numerous proteolytic enzymes results in the degradation of proteins during the isolation process (Wiemken *et al.*, 1979). Another problem is associated with the production of polyclonal antisera against glycoproteins, even after endomannosidase treatment, because residual carbohydrate residues provoke a strong antigenic response (Harlow and Lane, 1988). These problems can be circumvened by exclusive adsorption purification of the sera with carbohydrate columns, or yeast strains deleted for the *PRC1* gene. The latter has been attempted for polyclonal anti-CPY antiserum raised against purified yeast CPY available in this laboratory. Even after these procedures the unsatisfactory affinity of the antiserum to CPY and non-specific antigen binding to proteins other than CPY as judged by Western blotting and immunoprecipitation (results not shown) made the antiserum unsuitable for this study.

Glycosylation and contamination of an antigen with other proteins from the same organism can be prevented by expressing the protein as a fusion in *E. coli*, if the structural gene and sequence encoding the protein are known. One expression system that allows the expression of foreign proteins in *E. coli* is that of Muchmore *et al.*, 1989, which uses P_{lac} P_{tac} promoters linked to the pUC19 multiple cloning site and a transcriptional terminator region. Recombinant proteins are expressed as fusions with just 8 N-terminal amino acids from T4 lysozyme when induced by IPTG. The recombinant proteins expressed do not necessarily need to be purified to a large extent if in subsequent steps immunodetection of proteins in *E. coli* is not required.

An approximately 900 bp *Bam*HI fragment from *PRC1* has been cloned into the *E. coli* expression vector pEXP2 (Raymond *et al.*, 1990) to yield CPY protein expression plasmid pCKR108. The plasmid was a kind gift of Dr. Chris Raymond

(University of Oregon, USA) and was used in this study to express CPY antigen in *E. coli*. The corresponding protein was purified as described by Roberts *et al.*, 1989, and used to raise polyclonal antisera in New Zealand white rabbits.

3.2. Results

3.2.1. Induction of CPY Antigen Expression in MC1061 (pCKR108)

The recombinant CPY antigen was expressed in *E. coli* MC1061 harbouring the plasmid pCKR108. The plasmid carries the 900 bp *Bam*HI fragment from *PRC1* fused in frame into the *Bam*HI site of the multiple cloning site of pEXP2. Expression was induced by the addition of IPTG to a final concentration of 2 mM and the corresponding protein was purified as described in Materials and Methods and could be recovered in the Triton X-100 washed pellet (Figure 3.1). A total of 2.5 mg recombinant protein which had a size of about 43 kDa was recovered from a 500 mL culture. It was contaminated with low molecular weight proteins from *E. coli* which were successfully removed after electrophoresis on a preparative SDS-PAGE (Figure 3.2).

3.2.2. Production of Polyclonal Antiserum Against CPY

Two female New Zealand white rabbits were injected with SDS denatured CPY antigen and it was boosted after 12 and 34 days from the initial injection. Test bleeds were performed after 18 and 45 days and the titer of anti-CPY specific antibody determined in an ELISA using recombinant CPY and BSA coated 96 well cluster plates. The specific antibody binding was determined by subtracting the non-specific binding of each antiserum dilution to BSA from the binding of the same dilution to the original antigen, CPY. Since equal amounts of both specific and non-specific antigen were used in each assay, the absorbance values obtained after developing the ELISAs were directly comparable, assuming that both antigens bound to the plates with the same efficiency. The ability to bind antigen was dependent on the dilution of the antiserum

and was different for sera obtained after the first and second booster injections. For antiserum obtained after the first booster the specific binding activity steadily increased from a $1:1x10^{-6}$ dilution to reach an optimum at a 1:1,000 dilution (Figure 3.3A). A further increase in the antiserum concentration resulted in a decrease in specific antigen binding activity. This was due to an increase in non-specific binding of the antibodies to BSA at a higher concentration rather than to a decrease of binding activity to CPY, since the specific antigen binding reached a saturation level at about the same concentration (data not shown). The specific antigen binding activity of diluted antisera obtained after the second booster increased from a $1:10^{-4}$ dilution to a 1:10 dilution, and appears to be saturated at about 1:20 (Figure 3.3B). Rabbit B had a marginally higher antibody titer than rabbit A. The different response of antigen binding is due to a lower non-specific binding activity of the antisera rather than an increase in absolute binding (data not shown).

The antigenic responses of the two rabbits were comparable (Figure 3.4). The specific responses after the first booster were reduced after the second booster at a 1:1000 dilution (Figure 3.4), but the specificity of the sera after the second booster was increased significantly at higher antisera concentrations.

Antigen specificity was determined on a Western blot using commercial CPY and total yeast extracts as antigens (Figure 3.5). Both rabbits produced a highly specific polyclonal antiserum which only detected one CPY band on a Western blot in total yeast extract and several bands in a commercial CPY preparation. Immunoprecipitation of CPY in cell and supernatant fractions of Tran[^{35}S] labelled yeast sphaeroplasts with pre-immune serum obtained from a rabbit not immunized with CPY and 1 µL of serum from rabbit A detected a 61 kDa radiolabelled protein in the cell fraction only when immune but not when pre-immune serum was used (Figure 3.6). This is characteristic for a specific antigenic response to the CPY antigen. Rabbits were terminated after 46 days from the initial injection by cardiac puncture, the serum was separated from the clotted erythrocytes and aliquots of the sera were stored at -84^{o}C. A total volume of 20 mL serum from rabbit A and 23 mL serum from rabbit B were obtained.

Figure 3.1: Expression and purification of IPTG induced recombinant CPY in *E. coli*. Lanes 1, 3, 5 and 7 represent *E. coli* MC1061 harbouring pEXP2 as control; lanes 2, 4, 6 and 8 represent MC1061 harbouring the pCKR108 CPY recombinant fusion plasmid. No IPTG induced recombinant CPY is detectable in a total cell extract from the pCKR108 containing strain (Lanes 1 and 2). A slightly more abundant protein of approximately 43 kDa is detectable in the first supernatant from the strain harbouring the recombinant plasmid (Lane 4) but not in MC1061 (pEXP2). This protein band does not appear in the second supernatant from either strain (Lanes 5 and 6). However, a 43 kDa protein band is highly enriched in the Triton X-100 washed pellet from MC1061 (pCKR108; lane 8) but not in the strain harbouring the control plasmid (lane 7). Since the 43 kDa protein product is absent from a control and present in the Triton X-100 washed pellet of an IPTG induced strain with the recombinant plasmid it is likely that it represents recombinant CPY.

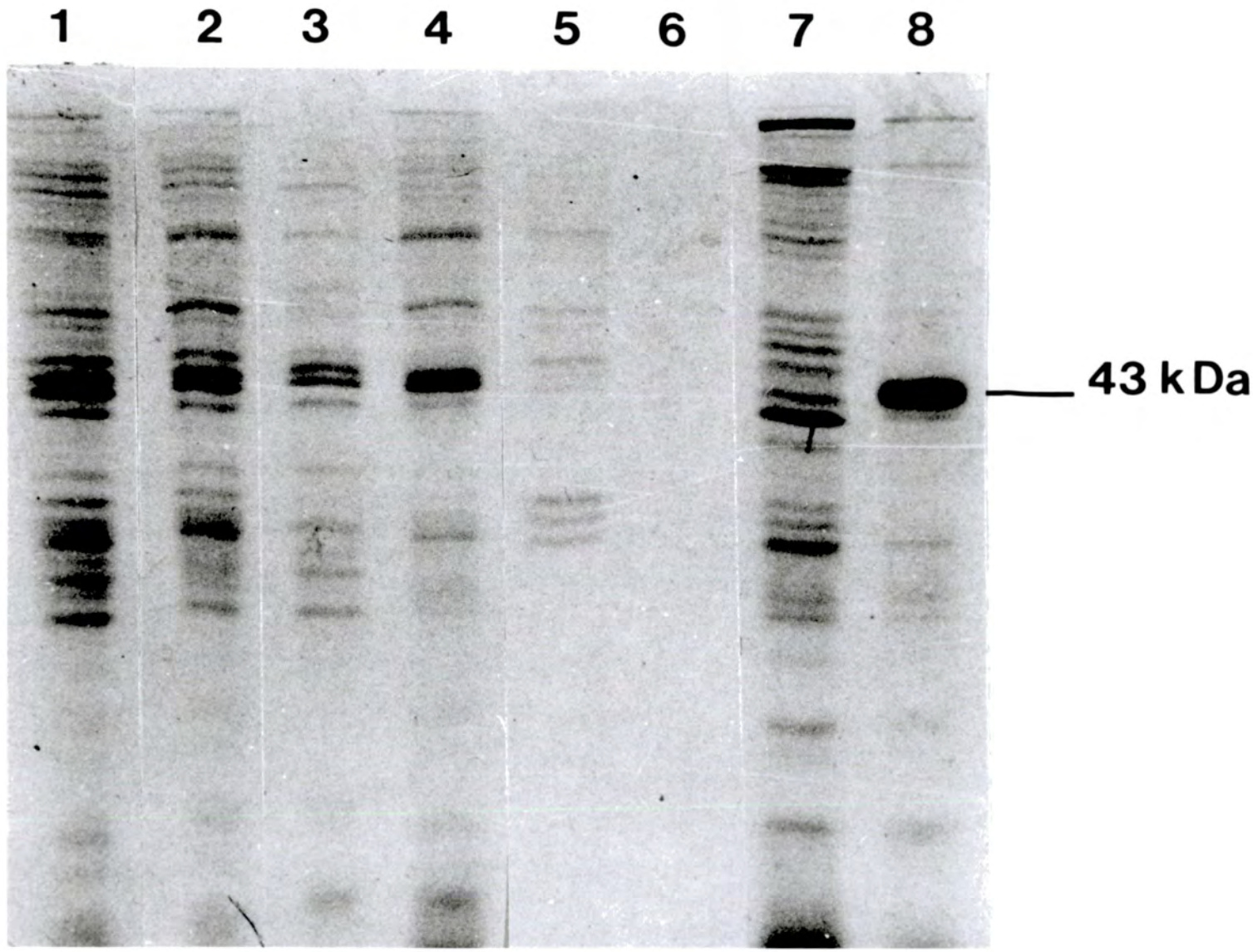

1
2
3
4
5
6
7
8
43 kDa

Figure 3.2: Large scale induction of recombinant CPY and SDS PAGE gel purification of the 43 kDa protein species. A 5 μg protein sample recovered from the Triton X-100 pellet of an IPTG induced MC1061 (pCKR108) strain was heavily contaminated with low molecular weight proteins (Lane 1) which successfully were removed after preparative SDS PAGE, excision and SDS elution of the appropriate band (Lane 2; 0.5 μg protein). Lane 3 contains low molecular weight markers representing 66, 45, 36, 29, 24 and 20.1 kDa from the start of the gel to the front. The excised and SDS eluted protein was pure and free of contaminant protein and was used without further purification for immunizations.

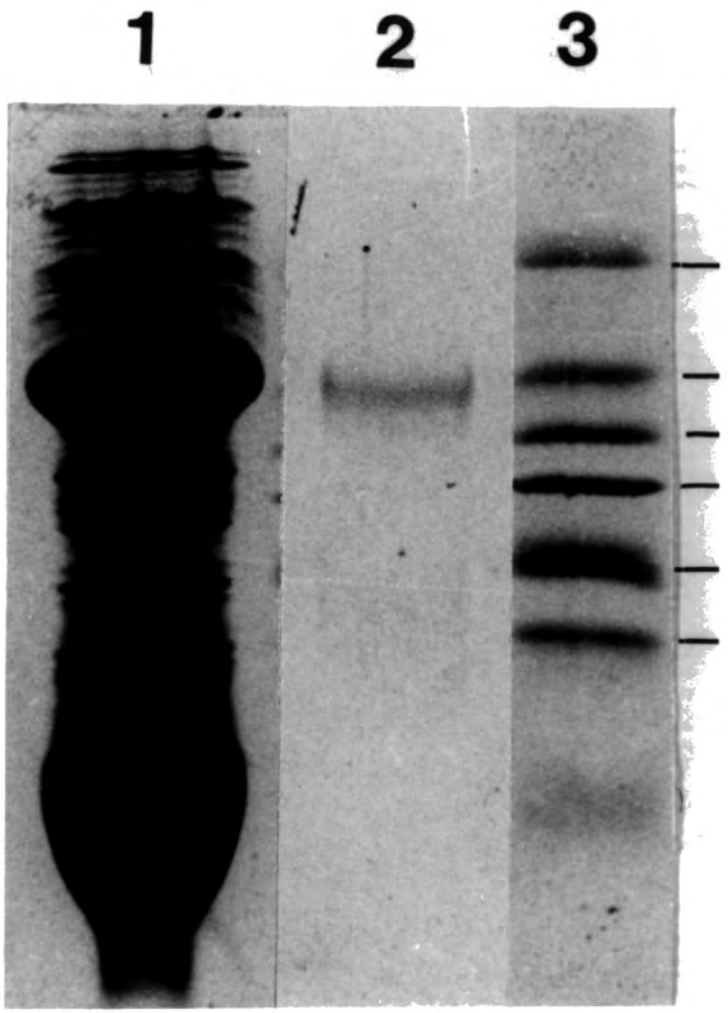
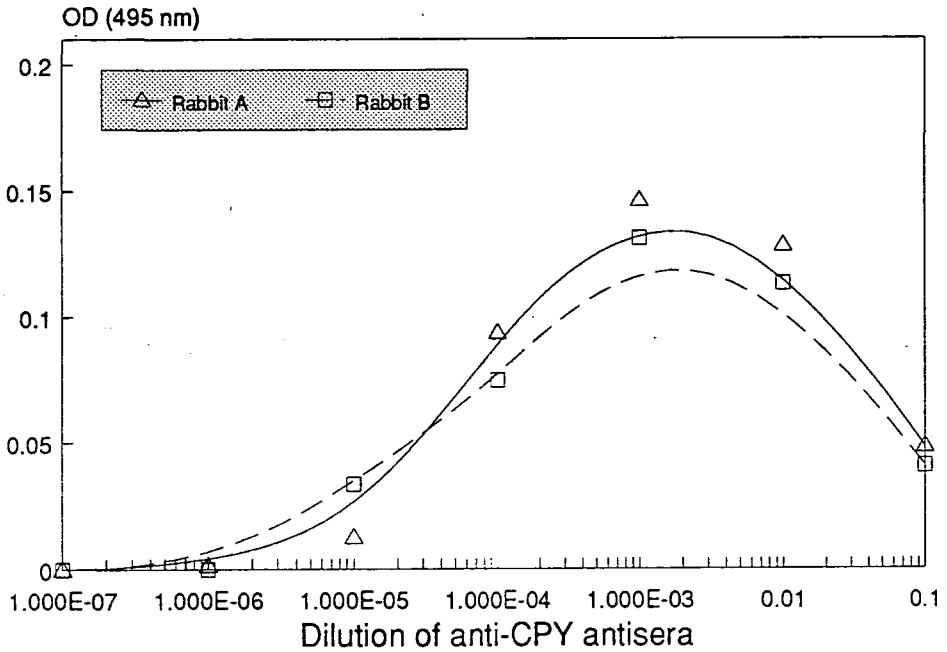
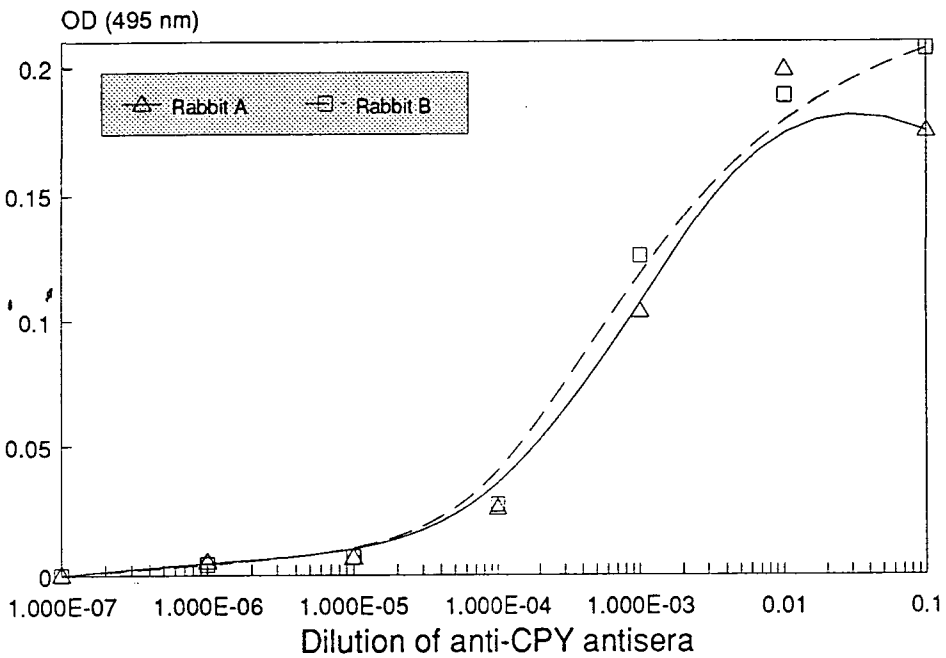

1
2
3

Figure 3.3: Optimal specific antigen binding activity of diluted antisera. Different dilutions of antisera obtained from rabbits A and B 12 days after the first booster injection (A) and 8 days after the second booster (B) were subjected to an ELISA using BSA (non-specific binding) and recombinant CPY at 0.16 ng protein per well as antigens. The absorbance of each dilution was determined at 495 nm 30 minutes after starting the peroxidase assay reaction and was a direct measure of the specific antigen binding of the antisera after substraction of non-specific (BSA) binding and a control reaction omitting the secondary anti-rabbit Ig-peroxidase conjugate antibody.


OD (495 nm)
0.2
0.15
0.1
0.05
0
Rabbit A
Rabbit B
1.000E-07
1.000E-06
1.000E-05
1.000E-04
1.000E-03
0.01
0.1
Dilution of anti-CPY antisera

A


OD (495 nm)
0.2
0.15
0.1
0.05
0
Rabbit A
Rabbit B
1.000E-07
1.000E-06
1.000E-05
1.000E-04
1.000E-03
0.01
0.1
Dilution of anti-CPY antisera

B

Figure 3.4: Specific antigenic response of a 1:1,000 antiserum dilution after the first and second booster injections. Antisera from both rabbits obtained 12 days after the first and 8 days after the second booster injection were subjected to an ELISA to determine the levels of specific antigen binding. BSA (non-specific binding) and recombinant CPY antigens were used at 0.16 ng protein per well. The absorbance of each well was determined at 495 nm 30 minutes after starting the peroxidase assay reaction and was a direct measure of the specific antigen binding of the antisera after substraction of non-specific (BSA) binding, and a control reaction omitting the secondary anti-rabbit Ig-peroxidase conjugate antibody.

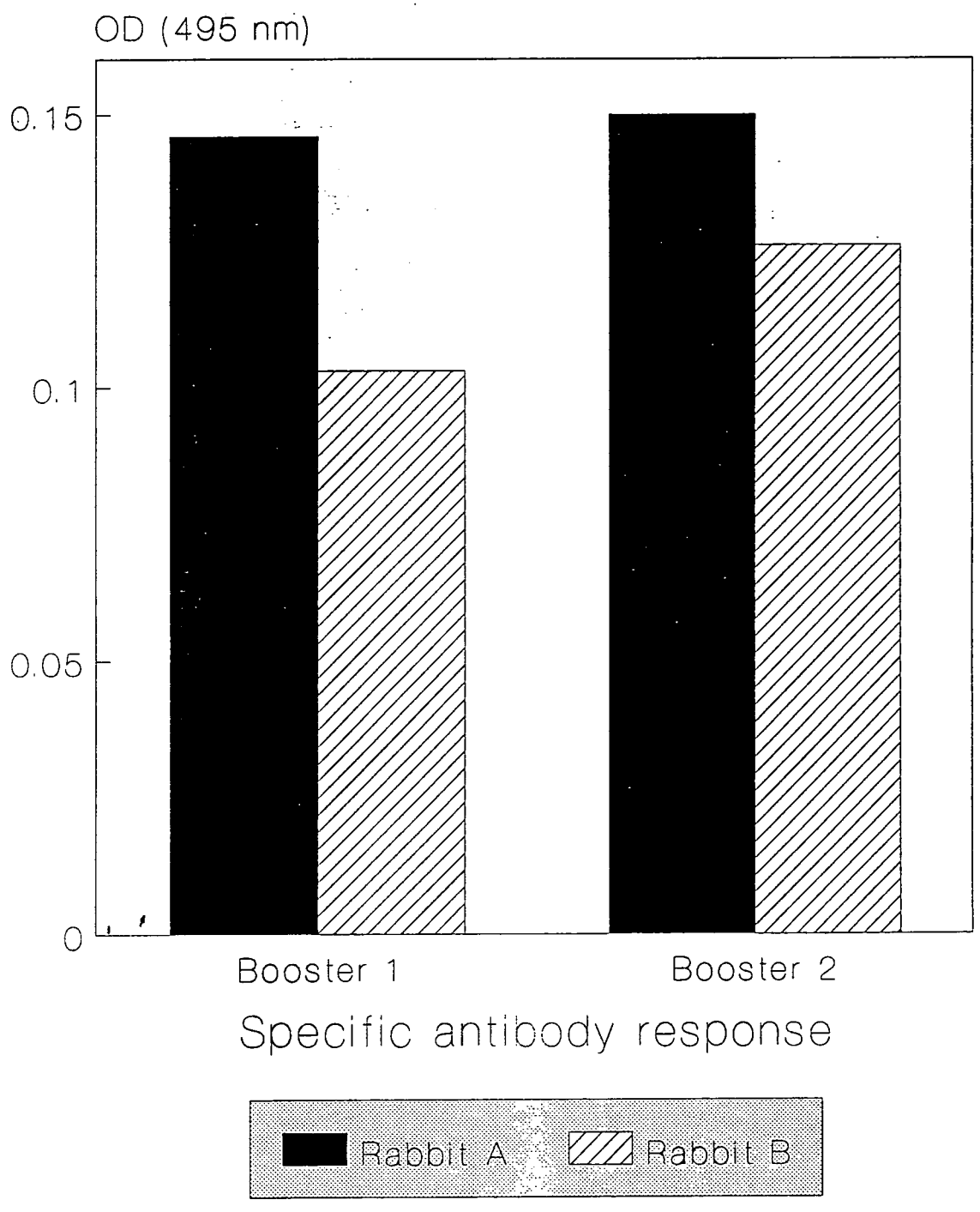

OD (495 nm)
0.15
0.1
0.05
0
Booster 1
Booster 2
Specific antibody response
Rabbit A
Rabbit B

Figure 3.5: Immunoblots of commercial CPY and total yeast cell extract with antisera from rabbit A and rabbit B. The equivalent of 1 μg commercial CPY (Sigma; lane 1) and 5 μg total yeast extract from strain SEY6210 (Lane 2) were separated on a 7.5% SDS PAGE and blotted onto nitrocellulose. The Western blot was developed using 0.1 μL/mL anti-CPY antiserum from rabbit A. The antibody recognizes several bands in a commercial CPY preparation, the more prominent band migrating at a molecular mass of 61 kDa (Lane3). The smaller size bands probably represent breakdown products of CPY. A single 61 kDa protein is recognized in total yeast extract from strain SEY6210 (Lane 4). The equivalent of 5 μg and 0.05 μg representing total yeast extract were separated on a 7.5% SDS PAGE and blotted onto nitrocellulose (Lanes 5 and 6, respectively). The Western blot was developed using 0.1 μL/mL anti-CPY antiserum from rabbit B as primary antibody. The antibody recognizes a single 61 kDa protein in both corresponding lanes (Lanes 7 and 8).

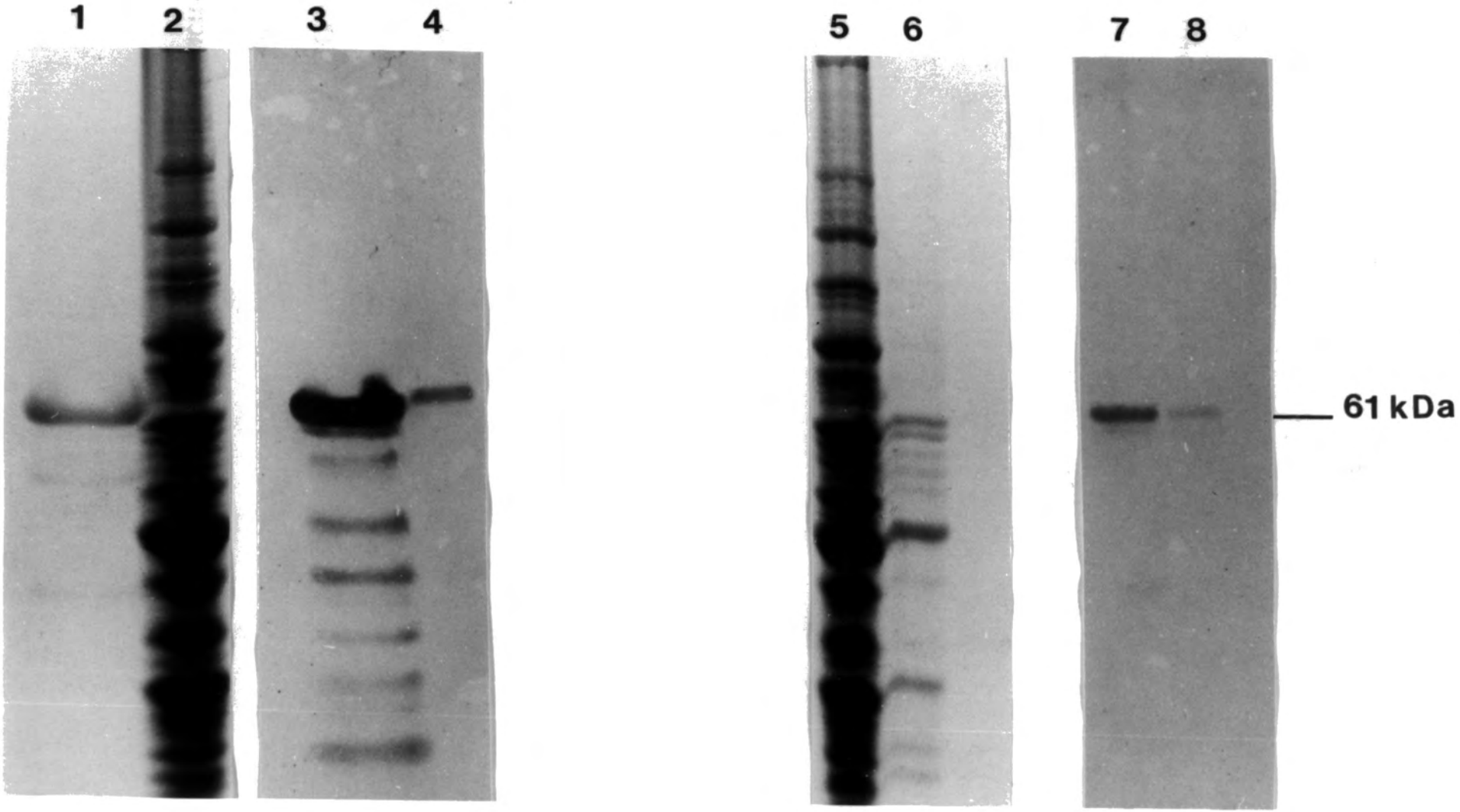
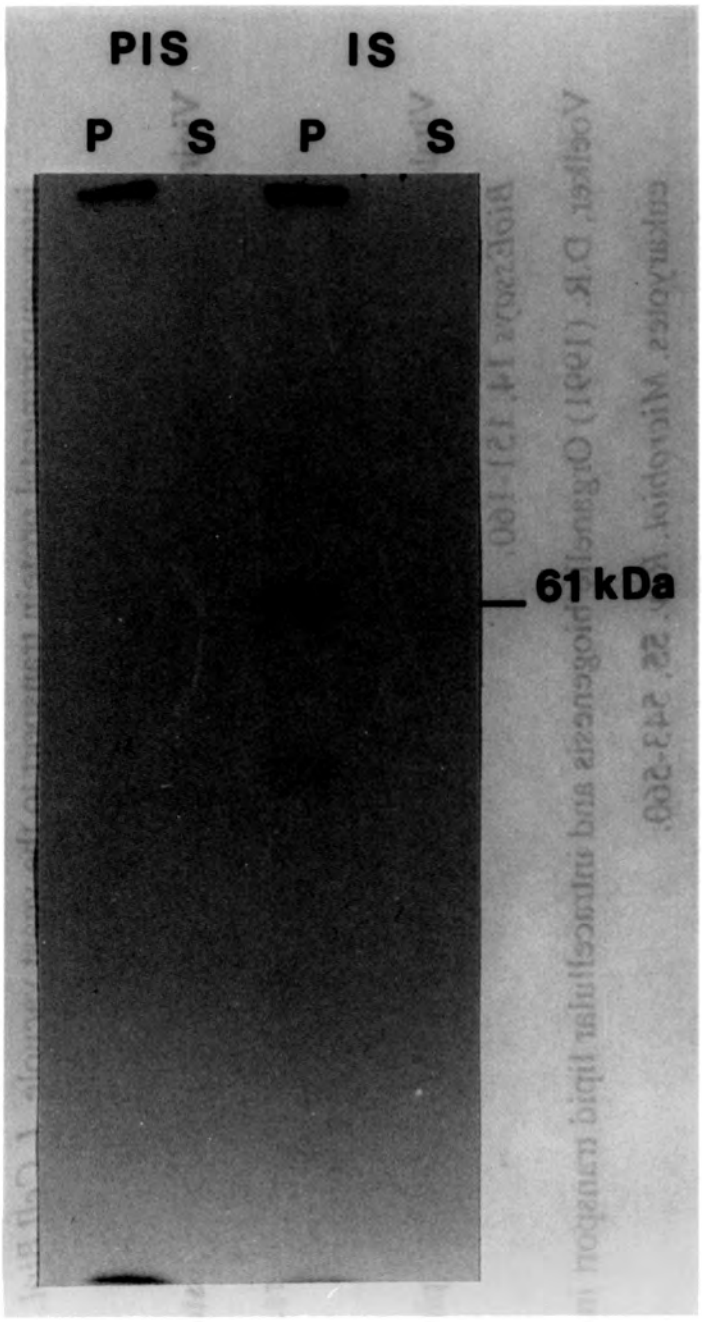

1
2
3
4
5
6
7
8
61 kDa

Figure 3.6: Immunoprecipitation of pulse-chase labelled CPY present total yeast extract and culture supernatant with pre-immune serum and antiserum from rabbit A. Strain SEY6210 sphaeroplasts were labelled with Tran[^{35}S] label and chased with cold methionine for 20 minutes. Both pellet (P) and supernatant (S) fractions were divided and incubated with 1 µL pre-immune serum (PIS) and 1 µl immune serum (IS) to immunoprecipitate radiolabelled protein. While the pre-immune serum does not recognize any radiolabelled protein in pellet and supernatant fractions, the anti-CPY serum from rabbit A recognizes a single 61 kDa protein in the pellet but not the supernatant fraction from wild type yeast.


PIS
IS
P
S
P
S
61 kDa

3.3. Discussion

Two polyclonal antisera against recombinant CPY expressed in *E. coli* were obtained from two rabbits. They had a high antigen binding titer to recombinant CPY and recognized one antigen of a molecular weight of 61 kDA in total yeast extracts on Western blots and in immunoprecipitations of pulse-chase labelled wild type yeast cells. The antisera also recognized commercial CPY which migrated at the same location on Western blots. This provides sufficient evidence to assume that the antisera are specific to CPY. The isolation of the antigen expressed in *E. coli* was unproblematic and the antibody of far better specificity than could have been obtained by using antigens isolated according to convenional protein purification procedures. It was decided not to further purify the antisera by ammonium sulfate precipitation and dialysis or by affinity purification because the sera were highly specific as judged by ELISA and Western blotting. Furthermore, immunoglobulins are generally less stable after purification, and affinity columns tend to bind the highest affinity antibodies irreversably (Harlow and Lane, 1988).

It was decided not to use the traditionally recommended methods of immunodiffusion and other immunoelectrophoretic techniques to determine the antibody titer and mono-specificity of the antisera (Clausen, 1981). The Ouchterlony antibody-antigen diffusion technique is commonly employed to assess the titer of antibodies in test-bleeds, but the technique is hardly quantitative, requires large amounts of antigen and can take up to 5 days until precipitin lines are visible. Additionally, not all antibody-antigen interactions lead to detectable pecipitin lines. The ELISA technique used in this study can easily be quantified, and is more sensitive in that it can detect lower antibody titers. For example, a typical Ouchterlony plate displays visible precipitation of the antibody-antigen complex in a range from 1:10 to 1:1 fold dilutions of antiserum using 5 μg antigen (C.K. Raymond, open citation). The ELISA described here was able to detect antibodies in a 1:10,000 fold dilution of antiserum using 0.16 ng antigen, and also could assess non-specific binding. The antisera were also tested for

specificity under experimental conditions such as Western blotting and immunoprecipitations. The experimental results clearly indicate that the antisera exclusively recognize CPY but not other antigens present in total yeast extract.

The antiserum from rabbit A has successfully been used in subsequent non-lysing colony hybridizations, Western blots and immunoprecipitations in this study, initially together with a small batch of affinity purified anti-CPY serum kindly provided by Tom Stevens (University of Oregon, USA), which was of equal purity and affinity. In immunoprecipitations if Immunosorb is used to retrieve antibody bound CPY, the antiserum from rabbit A will precipitate some proteins of lower molecular weight than the different forms of CPY (results not shown). These bands are easily removed if care is taken to resuspend the Immunosorb pellet thoroughly in the wash buffer.

Samples of antisera taken after the first and second booster reaction differed in their specific binding to CPY. Although the antibody titer of the two test-bleeds was lower after the second test-bleed, the specificity of binding at higher antibody concentrations was increased after the second booster injection. These findings are consistent with the current understanding of hyperimmunizations (Harlow and Lane, 1988), where the average affinity of antibodies for a particular antigen increases with repeated injections. B-lymphocyes expressing the highest affinity antigen receptors will compete most successfully for low concentrations of antigen. Clones of B-lymphocytes secreting higher-affinity antibodies will thus be selected for proliferation. Since somatic mutations of variable region genes occur, there will be a selective pressure for higher affinity antibodies. The antisera produced after the second booster injection appear to represent the higher-affinity antibodies. Since only relatively few higher affinity antibody secreting B-lymphocyte clones are selected, the overall titer of the antibodies might be lower, but this is compensated by improved specificity of the antisera.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF SALT-SENSITIVE VACUOLAR MUTANTS IN YEAST

4.1. Introduction

The yeast vacuole is an important acidic subcellular compartment involved in the homeostasis and storage of metabolites, including amino acids, polyphosphates, carbohydrates and ions. It contains a variety of hydrolytic enzymes which play an important role in proteolytic processing and intracellular macromolecular degradation, especially associated with nitrogen metabolism, the cell cycle and cellular control (Achstetter and Wolf, 1985; Klionsky *et al.*, 1990; Raymond *et al.*, 1992). These features make the yeast vacuole show some homology to both mammalian lysosomes (Wiemken *et al.*, 1979; Pisoni and Thoene, 1991) and plant vacuoles (Matile, 1978). Another function of the vacuole appears to be osmoregulation (Banta *et al.*, 1988) and the maintenance of cytosolic ion and pH homeostasis (Klionsky *et al.*, 1990). The vacuole can occupy 25 to 50% of the yeast cell and is known to undergo dynamic changes in response to growth conditions (Raymond *et al.*, 1992). The vacuole does not have its own genome as for example mitochondria and it has to derive all its protein constituents directly from the cytoplasm (Kane *et al.*, 1989a; Yoshihisa and Anraku, 1990), as well as via the secretory pathway (Klionsky *et al.*, 1990; Raymond *et al.*, 1992; Stevens *et al.*, 1982; Vida *et al.*, 1991) and the endocytotic pathway (Chvatchko *et al.*, 1986; Jennes and Spatrich, 1986; Makarow, 1985a, 1985b; Riezman, 1985). Some recent experimental evidence also suggests that proteins can enter the vacuole directly for degradation (Chiang and Schekman, 1991). This is thought to occur by a mechanism where proteins destined for vacuolar degradation enter peroxysomes which then are localized into the vacuole via some novel mechanism (H.-L. Chiang, personal communication). The vacuolar morphology is plastic and depends very much on

external conditions such as the presence of nutrients or the cell cycle (Gomes de Mesquita *et al.*, 1991; Pringle *et al.*, 1991; Wiemken *et al.*, 1970). During mitosis, the vacuole buds off some of its material, which is transported into the daughter bud (Weisman and Wickner, 1987; Weisman *et al.*, 1990) suggesting a mechanism for vacuole derived vesicle formation, scission and transport into the daughter bud. This is associated with some characteristic segregation pattern in the form of large vesicles or tubular structures (Gomes de Mesquita *et al.*, 1991; S.D. Emr and T. Vida, personal communication; M.D. Watson, unpublished observation). Unlike for example the Golgi in mammalian cells, which cannot be synthesised *de novo* (G.R. Warren, open citation), vacuole biosynthesis does apparently not depend on inherited vacuolar material (Banta *et al.*, 1988), although the experimental evidence suggesting *de novo* biogenesis is indirect.

Table 4.1 lists a selection of the most important constituents of the yeast vacuole currently known. Whereas much information about most inorganic ions and storage metabolites exists, little is known about the vacuolar protein constituents. Only about 10 to 20 vacuolar proteins have been described in the literature, whereas there are about 100 proteins detectable by Coomassie staining of SDS-PAGE separated Na_2CO_3 washed vacuolar membranes alone (M.D. Watson, unpublished data). Little is known about the function of most proteins with the exception of a few vacuolar proteinases and the vacuolar membrane ATPase which have been studied in detail (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). In the following few sections an attempt is made to review various aspects of vacuole biogenesis according to the constituents involved, their synthesis and processing and their import pathway into the vacuole. Simultaneously, the present knowledge of organelle inheritance as well as the role of the vacuole as cellular compartment is summarized. However, the main theme to consider always is that the yeast vacuole is one organelle which carries out a number of overlapping, often related functions in concert, and the focus on only one aspect at a time can easily distort the actual image of vacuole maintenance and biogenesis.

Constitutent	Function	Reference
Ions		
H^+	Acidification	Kane *et al.*, 1989a
Na^+	?	Cramer and Davis, 1984
K^+	Osmoregulation (?)	Okorokov *et al.*, 1980
Mg^{2+}		Okorokov *et al.*, 1980
Ca^{2+}	Detoxification, storage	Ohsumi and Anraku, 1983
Zn^{2+}	Cofactor (?) (API)	White and Gadd, 1987
Sr^{2+}	Detoxification	White and Gadd, 1986
Co^{2+}	Detoxification (?)	White and Gadd, 1986
Pb^{2+}	Detoxification	White and Gadd, 1986
P_i	Storage; charge balance	Urech *et al.*, 1978
Amino Acids		
Arginine	Nitrogen storage	Kitamoto *et al.*, 1988c
Asparagine	Storage	Kitamoto *et al.*, 1988c
Glutamine	Storage	Kitamoto *et al.*, 1988c
Histidine	Storage	Kitamoto *et al.*, 1988c
Isoleucine	Storage	Kitamoto *et al.*, 1988c
Leucine	Storage	Kitamoto *et al.*, 1988c
Lysine	Storage, detoxification	Kitamoto *et al.*, 1988b
Phenylalanine	Storage	Cooper, 1982
Tryptophan	Storage	Cooper, 1982
Tyrosine	Storage	Kitamoto *et al.*, 1988c
Metabolites (?)		
Trehalose	Stress protectant	De Virgilio *et al.*, 1991
Soluble Proteins		
Aminopeptidase I	Proteolysis	Frey and Röhm, 1978
Aminopeptidase Co	Proteolysis	Achstetter and Wolf, 1985
Carboxypeptidase S	Proteolysis	Achstetter and Wolf, 1985
Carboxypeptidase Y	Proteolysis	Achstetter and Wolf, 1985
Polyphosphatases	Degradation	Wiemken *et al.*, 1979
Proteinase A	Specific proteolysis	Schwencke, 1991
Proteinase B	Specific proteolysis	Schwencke, 1991

Constitutent	Function	Reference
Soluble Proteins		
RNAse	?	Schwencke, 1991
Trehalase	Trehalose degradation	Schwencke, 1991
Membrane Associated Proteins		
α-mannosidase	?	Opheim, 1978
Alkaline phosphatase		Wiemken *et al.*, 1979
AMPase	?	Wiemken *et al.*, 1979
ATPase (7 subunits)	Acidification	Kane *et al.*, 1989a
DPAP B		Roberts *et al.*, 1989

Table 4.1: Major constituents of the yeast vacuole.

4.1.1. Vacuolar Ion Transport and Storage

The regulation of the cytosolic ion concentration in yeast as in any other cell is necessary, because some ions, such as Co^{2+}, Pb^{2+} and Sr^{2+} are protein inhibitors and need to be removed from the cytosol (White and Gadd, 1986). Ions like Ca^{2+}, Mg^{2+} and Zn^{2+} are required in physiological concentrations, but can be toxic to the cell when that concentration is exceeded (Cornelius and Nakashima, 1987; Ohya *et al.*, 1986; Raguzzi *et al.*, 1988). Furthermore, the concentration of Ca^{2+} has to be regulated because of its involvement in several regulatory processes and signalling pathways (Carafoli, 1987). Also, the vacuole has to import protons required for the acidification and certain transport processes coupled to the release of protons (Okorokov *et al.*, 1985). The yeast vacuole plays a prominent role in the selective sequestration and regulated release of the above mentioned ions (Klionsky *et al.*, 1990). The vacuole of *Neurospora crassa*, which is believed to have similar features to the *S. cerevisiae* vacuole, is impermeable to a variety of mono- and bivalent ions. Uptake of Mg^{2+}, Ca^{2+}, Na^{+} and K^{+}, as well as basic amino acids, is highly selective and evidence suggests the involvement of an active process (Cramer and Davis, 1984). Recent evidence points towards an H^{+} antiport involved in the selective uptake of Ca^{2+} (Ohsumi and Anraku, 1983; Ohsumi *et al.*, 1988) and Zn^{2+} (Okorokov *et al.*, 1985) in *S. cerevisiae*. Other cations are also taken up, but their transport properties have not been established yet (Okorokov *et al.*,

1980, 1985; White and Gadd, 1986). It appears that the yeast vacuole is the main compartment ensuring homeostasis of Ca^{2+} ions in the cytoplasm of yeast cells (Ohsumi and Anraku, 1983). A central role in vacuole acidification and uptake of other ions is played by the vacuolar membrane H^+-ATPase, whose activity is stimulated by the presence of Ca^{2+} and Mg^{2+} (Ohsumi and Anraku, 1981; Ohsumi and Anraku, 1983) or other ions (Okorokov *et al.*, 1985). The ability of the vacuole to store other metabolites and ions depends on presence of a proton gradient (Klionsky *et al.*, 1988). In other eukaryotes the acidification of the vacuolar network is required for a variety of cellular processes, such as receptor-mediated endocytosis, protein sorting and targeting, activation of zymogens and the assembly of secretory granules (Mellman *et al.*, 1986). Except zymogen activation, which in yeast does not depend on an acidic vacuole (Klionsky *et al.*, 1992), other processes like efficient protein sorting to the vacuole (Klionsky *et al.*, 1992), and amino acid and ion transport (Klionsky *et al.*, 1990) depend on acidification. The vacuolar ATPase requires ATP as substrate for proton uptake (Ohsumi and Anraku, 1981).

K^+ might function as regulator of vacuolar ion pools (Ohsumi *et al.*, 1988) by maintaining the charge balance and triggering the release of other ions like Ca^{2+} or arginine (Eilam *et al.*, 1985). A membrane potential-dependent cation channel capable of conducting K^+ and other monovalent cations has been characterized by an electrophysiological method (Wada *et al.*, 1987; Tanifuji *et al.*, 1988). It appears that the channel is regulated by the membrane potential and the presence of Ca^{2+} on the cytoplasmic site.

The major anion present in the vacuole is inorganic phosphate (P_i) in the form of a polymer, polyphosphate (Cramer and Davis, 1984; Urech *et al.*, 1978), although the mechanism of uptake of the monomer and regulated polymerisation in the vacuole remains obscure. It is believed that polyphosphate is involved in the retention of basic amino acids and Ca^{2+} in the vacuole, although not exclusively, as Ca^{2+} can be readily released upon the addition of H^+ or Ca^{2+} ionophores (Ohsumi and Anraku, 1983). It seems more likely that polyphosphate serves as a pimary store for phosphates in an osmotically inactive form, and as an overall charge balancing agent (Cramer and Davis,

1984), especially since it has been demonstrated that the vacuolar polyphosphate can polymerize and depolymerize rapidly (Langen *et al.*, 1962; Pisoni and Lindley, 1992). The presence of a polymerized anion will help to balance the charge, and reduce the osmotic potential as would not be possible by monomeric anions. On the other hand, it has been demonstrated that when Ca^{2+} is sequestered in the vacuole in large amounts a Ca^{2+}-polyphosphate inclusion was formed (Ohsumi *et al.*, 1988). The vacuole appears to be the only compartment in yeast which accumulates polyphosphate (Urech *et al.*, 1978; Shanks and Bailey, 1990). The presence of an endopyrophosphatase and an exopyrophosphatase in the vacuole (Schwencke, 1991) suggests that its activity is regulated by some mechanism to avoid depolymerization of pyrophosphate under undesirable condition. There is no coherent knowledge about other anions inolved in maintaining the overall charge balance of the vacuole, but indirect evidence suggests that other anions must maintain this charge balance because polyphosphates only represent at most 75% of the anions present in the vacuole (Cramer and Davis, 1984). Also during the sudden release of some Ca^{2+} the polyphosphate pool does not undergo measurable change (Ohsumi and Anraku, 1983) arguing for the existence of another mechanism for rapid charge balance.

The selective uptake of ions by the vacuole and their regulated release requires ion channels as well as a regulatory mechanism. The transmembrane subunits of the channel complexes have to be targeted to the vacuole. This requires mechanisms of targeting these proteins to the vacuolar membrane in the correct orientation. The selective sequesteration or release of ions also requires a regulatory mechanism that senses cytoplasmic ion concentrations and activates or deactivates channels according to the requirements of the cell. The vacuolar protein constituents contributing to ion storage and maintenance of ion gradients are described in later sections.

4.1.2. Amino Acid Storage

Comparatative analysis of cytoplasmic and vacuolar amino acid pools suggests that the yeast vacuole functions as a store for a number of amino acids (Kitamoto *et al.*,

1988c). Kinetic studies on isolated vacuolar vesicles demonstrated that eight independent amino acid transporter systems exist (Sato *et al.*, 1984b). Transporters for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine and isoleucine-leucine are H^+ antiport systems (Sato *et al.*, 1984b). An additional arginine-histidine exchanger that utilizes the chemical potential of a histidine gradient also exists (Sato *et al.*, 1984a). Analysis of the vacuolar amino acid pools is in good agreement with the presence of the above amino acid transport systems (Kitamoto *et al.*, 1988c). The sequesteration of certain amino acids in the vacuole is consistent with the idea that the vacuole acts as a storage organelle to allow survival of the yeast cell under unfavourable conditions such as nitrogen starvation. This is particularly the case for arginine which can reach intravacuolar concentrations of 430 mM. Indirect evidence for this hypothesis comes from the examination of the vacuole deficient mutant *end1* (*vps11*). The mutant does not accumulate any polyphosphate or arginine, and as a consequence growth of the mutant strain ceases much earlier than the corresponding wild type strain (Westenberg *et al.*, 1989). Another supplementary explanation for the accumulation of certain amino acids in the vacuole is the regulation of cytoplasmic amino acid concentrations. Kitamoto *et al.*, 1988c, have shown that the cytoplasmic concentrations of amino acids remain constant, even when the growth medium was supplemented with individual amino acids. Lysine and histidine cannot be degraded by *S. cerevisiae* (Watson, 1976), and the uptake of any excess lysine and/or histidine is necessary because an excess of these amino acids can be toxic to the cell (Kitamoto *et al.*, 1988b). This strategy has been employed to isolate mutants which are deficient in the vacuolar uptake of lysine (Kitamoto *et al.*, 1988b).

The presence of three independent arginine transport systems stresses the importance of arginine sequesteration in the vacuole. One of the three channels described is a H^+/arginine antiport driven by a proton motive force which is inhibited by Cu^{2+}, Ca^{2+} or Zn^{2+} (Ohsumi and Anraku, 1981). Arginine is the amino acid with the largest ammonium content per mole, and probably serves as the main nitrogen store of the yeast cell (Kitamoto *et al.*, 1988c; Klionsky *et al.*, 1990). Arginine represents about 30% of the basic amino acid pool in *S. cerevisiae* (Dürr *et al.*, 1979). The retention of

arginine and other basic amino acids, which under the vacuolar pH conditions are protonated, is thought to occur by polyphosphate acting as a cation trap, partly because of the stoichiometrical correlation between polyphosphate and arginine (Cramer and Davis, 1984; Dürr *et al.*, 1979). The fact that both pools can be regulated independently (Cramer and Davis, 1984) indicates that there is no absolute requirement for polyphosphates to trap arginine in the vacuole, and any association in a complex may function in reducing the osmotic potential of the vacuole. Retention of amino acids in the vacuole is due to the impermeability of the vacuolar membrane to amino acids and an active mechanism to take up amino acids from the cytoplasm (Klionsky *et al.*, 1990). Arginine is mobilized from the vacuole to the cytoplasm under certain nitrogen starvation conditions (Kitamoto *et al.*, 1988c) in a reaction that might require energy to allow arginine efflux (Drainas and Weiss, 1982). The mechanism of efflux and the signalling path to initiate release of stored ions or amino acids are poorly understood (Klionsky *et al.*, 1990).

4.1.3. Carbohydrate Storage

Relatively little is known about the role of the yeast vacuole in carbohydrate metabolism. The presence of a trehalase in the vacuole (Wiemken and Schellenberg, 1982) suggests that part of the trehalose metabolism is comparmentalized in the vacuole. Trehalose is a non-reducing disaccharide of glucose and is thought to be part of the primary heat shock response in *S. cerevisiae* and also confers resistance to dessication (Hottiger *et al.*, 1987). Trehalose accumulation in the cytoplasm is also believed to confer osmoresistance (Mackenzie *et al.*, 1988). Trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase are osmotically induced in *E. coli* (Kaasen *et al.*, 1992), but whether this is the case for *S. cerevisiae* is unknown and in addition trehalose might be located in the cytoplasm in *S. cerevisiae* (Keller *et al.*, 1982).

Some recent results suggest an intriguing involvement of the vacuole in carbohydrate metabolism. Plankert *et al.*, 1991, reported that the vacuolar alkaline

phosphatase (Pho8p) is identical to fructose-2,6-bisphosphate 6-phosphatase which converts fructose-2,6-bisphosphate to fructose-2-phosphate and P_i. The dephosphorylation of the 6-phosphate of fructose-2,6-bisphosphate is a key reaction in regulation of the carbohydrate metabolism in *S. cerevisiae* (Holzer, 1990). Fructose-2,6-bisphosphate is produced as an indirect response to the presence of glucose and regulates the switch from gluconeogenesis to glycolysis (Hers *et al.*, 1988). This reaction has to occur in the vacuole because of the unique localization of the *PHO8* gene product, and requires a transport mechanism for fructose-2,6-bisphosphate into the vacuole. An interesting question remains whether fructose-2-phosphate is released into the cytoplasm or remains vacuolar. Some mutants that mislocalize vacuolar proteins are defective in gluconeogenesis (Dulic and Riezman, 1989). This fact seems to suggest that the vacuole does regulate part of the gluconeogenetic pathway, and if the vacuole or part of it is defective other functions will be affected. So far no experimental approach has concentrated on this intriguing phenomenon.

4.1.4. Homeostasis Function of the Vacuole

The selective accumulation of ions and the presence of a number of transport channels support the hypothesis that the vacuole is a homeostatic organelle (Klionsky *et al.*, 1990). Its function in pH homeostasis has firstly been suggested by Banta *et al.*, 1988, who found that a vacuolar biogenesis mutants were sensitive to variation in the pH of the surrounding medium. The fact that the vacuolar as well as the cytoplasmic pH undergo relatively small changes in response to larger alterations of the pH in the environment has been attributed to the action of the vacuolar ATPase (Klionsky *et al.*, 1990). The fact that the plasma membrane ATPase is essential for growth under a number of unfavourable conditions including slight changes in environmental pH (McCusker *et al.*, 1987) argues against the functioning of the vacuole in cellular pH homeostasis. The vacuolar acidification will secondarily contribute to an increase in cytoplasmic pH, but its prime function is in maintaining the vacuole an acidic environment. The fact that mutants lacking a vacuole are osmosensitive, has been

suggested to be caused by the absence of an osmoregulatory organelle or vacuolar components involved in osmoregulation (Banta *et al.*, 1988; Klionsky *et al.*, 1990; Latterich and Watson, 1991). These hypotheses are supported by the fact that the vacuole contains osmotically active components, such as polyphosphate, arginine and K^+ (Klionsky *et al.*, 1990). The only conclusive evidence for a role of the vacuole in ion homeostasis has been demonstrated for Ca^{2+}. Mutants sensitive to elevated concentrations of Ca^{2+} in the growth medium are almost exclusively defective in some vacuolar function, including vacuolar acidification (Ohya *et al.*, 1986; Ohya *et al.*, 1991). The precise mechanism of ion uptake and release and charge balance is still poorly understood. The vacuole plays a similar role in amino acid homeostasis by selectively sequestering amino acids resulting in a constant concentration of these particular amino acids (Kitamoto *et al.*, 1988b; Kitamoto *et al.*, 1988c; Klionsky *et al.*, 1990). From the latter two examples it appears that the vacuole has the ability to selectively uptake components which would be toxic if not removed from the cytoplasm, and thus to tightly regulate the cytoplasmic concentration of these components. The regulation of the homeostasis mechanism is not understood, but judging from the number of constituents accumulating in the vacuole it might be quite sophisticated.

4.1.5. Vacuole Inheritance

The vacuolar compartment is usually inherited from the mother cell into the daughter bud as soon as the daughter bud emerges (Weisman *et al.*, 1987). Stable fluorophore labelling of the vacuolar compartment (Weisman and Wickner, 1988) and time lapse photography using confocal scanning laser microscopy (Gomes de Mesquita *et al.*, 1991) have been used to assess the inheritance of vacuolar material from mother cell to the bud. The inheritance pattern is distinct in a number of different wild type strains examined (Gomes de Mesquita *et al.*, 1991), but some general features do seem to exist. For the most commonly used wild type yeast strain X2180 the majority of buds aquire a vacuolar segregation structure within 15 minutes after bud emergence. The

separation structure has the form of a tubule or a vesicular stream (Gomes de Mesquita *et al.*, 1991; S.D. Emr and T. Vida, personal communication; C.K. Raymond, personal communication). It also is observed in semi-permeabilized cells (T. Vida, personal communication). The vacuolar segregation structure originates from the mother vacuole, elongates and moves across into the bud before the tubule or stream disappears. This process is completed within 20 minutes after initial bud emergence and before nuclear division occurs. The segregation pattern observed is not disturbed by the presence of nocodazole, a microtubule disrupting drug, suggesting that microtubules do not actively participate in segregation. Nocodazole does, however, disrupt vacuole integrity in X2180 (Guthrie and Wickner, 1988), but even that does not prevent the formation of segregation patterns. The inherited vacuole continues to grow during the cell cycle, presumably by *de novo* synthesis via the secretory pathway. In X2180 the observed disruption of the vacuolar compartment in the presence of nocodazole might be a consequence of a destabilized cytoskeleton which might participate in confining the vacuole as a single compartment (Guthrie and Wickner, 1988). The budding off of vacuolar vesicles might be mediated by a local destabilization of the vacuole surrounding cytoskeleton permitting segregation to occur.

4.1.6. Synthesis and Processing Pathway of Vacuolar Proteins

A great deal of interest has focused on understanding the pathway for the delivery of vacuolar proteins to the vacuole (Rothman *et al.*, 1989a; Stevens *et al.*, 1982), the activation of vacuolar zymogens (Mechler *et al.*, 1987), and the molecular mechanisms involved in the sorting process and vesicle budding and fusion (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). A great deal of information about the synthesis and transportation of proteins into the vacuole has been derived by studying the biogenesis of the soluble vacuolar protein carboxypeptidase Y (Stevens *et al.*, 1982). The fact that most vacuolar enzymes are glycoproteins (Hasilik and Tanner, 1978b) seems to suggest that these proteins share parts of the secretory pathway where glycosylation occurs (Esmon *et al.*, 1981; Esmon *et al.*, 1984). Since it is known that preproCPY (ppCPY) is

processed in two independent proteolytic events (Hasilik and Tanner, 1978a), the comparment dependent maturation of preproCPY was assessed (Stevens *et al.*, 1982). The isolation of conditionally lethal mutants in the secretory path, and the subsequent classification of the *sec* (*sec*retory) mutants into groups which define at least three distict linear compartments in the secretory pathway (Novick *et al.*, 1981; Deshaies and Schekman, 1987) showed that vacuolar proteins enter the secretory pathway and at some stage are sorted and routed to the vacuole. Different *sec* mutants interrupt transport in the secretory pathway at different steps at the non-permissive growth temperature (37°C), and accumulated material is discharged in most mutants to the next successive step upon return to the permissive temperature (24°C). The presence of pulse-labeled CPY was detected by immunoprecipitation of total cell extracts from mutants grown at the restrictive temperature. This includes those that block the import into the ER, the budding of transport vesicles from the ER or the subsequent fusion with the Golgi apparatus, mutants that block within or after the Golgi, and mutants that block the fusion of Golgi derived secretory vesicles with the plasma membrane (Deshaies and Schekman, 1987; Stevens *et al.*, 1982). Translocation deficient mutants *sec61* and *sec62* accumulated the unglycosylated signal peptide containing form of CPY, preproCPY (Hasilik and Tanner, 1978a; Deshaies and Schekman, 1987), in the cytoplasm. In the wild type the signal peptide is cleaved during translocation into the ER by a signal peptidase (Blachly-Dyson and Stevens, 1987; Johnson *et al.*, 1987). A thermoreversible accumulation of CPY precursors was detectable in mutants that block after the ER and the Golgi but not in mutants that block transport of secretory vesicles. Two CPY precursors were found to accumulate: p1CPY (67 kDa) accumulated at 37°C in *sec18*-1 and represents the core-glycosylated ER form of CPY, which also accumulates in all other ER blocked mutants. In *sec7* and *sec14* mutant strains, another CPY precursor (69 kDa) could also be immunoprecipitated. This precursor was designated p2CPY and represents the fully glycosylated Golgi precursor of CPY. Secretory vesicle blocked mutants did not accumulate significant amounts of precursors but produced mature CPY (61 kDa) at the restrictive temperature. The maturation sequence of the two CPY precursors was assessed with the help of double mutants which were *pep4* and either

sec18, *sec7* or *sec1*. *sec18* and *sec7* were epistatic to the *pep4* mutation and mainly produced p1CPY at 37°C. The p2CPY was observed in a *pep4* or a *pep4 sec1* double mutant. p1CPY thus precedes p2CPY. These pioneer experiments showed that the maturation and vacuolar targeting of CPY required the ER and Golgi and several gene products defining these compartments in yeast.

The increase in molecular mass of ppCPY to the ER form p1CPY is due to dolichol-mediated core glycosylation of the four Asn-X-Thr N-glycosylation sites. The added core oligosaccharides have the structure $P_2(GlcNac)_2Man_9Glc_3$ upon addition and are subsequently trimmed by glucosidase I and II and an α-mannosidase to yield the 67 kDa p1CPY (Esmon *et al.*, 1984). Phosphorylation of the carbohydrate side chains occurs in the ER, unlike for the secreted enzyme invertase where the phosphorylation event occurs in the Golgi compartment (Hashimoto *et al.*, 1981; Stevens *et al.*, 1982; Trimble *et al.*, 1983). p1CPY exits the ER via a vesicular intermediate transport carrier (Kaiser and Schekman, 1990; Rexach and Schekman, 1991) which is accumulated in restrictive *sec18* mutants because of their inability to fuse with the Golgi compartment (Wilson *et al.*, 1989). Upon fusion of the ER derived transport intermediates with the *cis* Golgi, p1CPY is released into the Golgi lumen. At some stage in the Golgi outer chain carbohydrate modification occurs (Franzusoff and Schekman, 1989) by the addition of α-1,6-mannose linked carbohydrates and subsequent addition of α-1,3-mannose linked carbohydrates. Compared to the secreted form of invertase, which is extensively outer-chain glycosylated on seven of nine glycosylation sites during migration through the Golgi ($Man_{>20}GlcNAc$), CPY receives relatively few carbohydrate side chains (Man_{11} to $Man_{18}GlcNAc$) on three of the four core oligosaccharides on preCPY (Trimble *et al.*, 1983). This difference is not due to the diffent nature of secretory and vacuolar enzymes or that the sorting of CPY from secretory enzymes occurs before extensive outer chain modification takes place. It rather is an intrinsic property of invertase and CPY, since preproCPY-invertase fusions which are correctly targeted to the vacuole in wild type strains receive extensive outer-chain mannose addition on the invertase moiety which are indistinguishable from the mature secreted invertase (Johnson *et al.*, 1987). Endo-β-N-acetylglucosaminidase H

(Endo H) digestion of native and denatured CPY and invertase revealed that one of the three high mannose oligosaccharides of CPY and two of nine oligosaccharides of invertase are inaccessible to Endo H treatment when in their native conformation. Denaturation of both proteins made all N-linked oligosaccharides accessible to Endo H treatment. The inaccessible N-linked oligosaccharides of both proteins have less outer chain mannose present and are devoid of phosphate which is associated with all the other oligosaccharides (Trimble *et al.*, 1983). The fully glycosylated p2CPY precursor has a molecular mass of 69 kDa and is believed to leave the Golgi at the *trans* compartment where sorting occurs (Graham and Emr, 1991). The sorting of soluble vacuolar proteins from proteins with other destinations is believed to occur by an active process, where sorting signals present in the pro sequence of vacuolar proteins are recognized. Proteins with such signals are packaged and sorted away from other proteins sharing the same Golgi compartment (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). Membrane associated vacuolar proteins share the same physical compartments as soluble vacuolar proteins, but for proteins with transmembrane domains the default pathway appears to be transport to the vacuole (Raymond *et al.*, 1992; T.H. Stevens, personal communication).

To date the transport step from the distal Golgi compartment to the vacuole is poorly defined, partly because of a lack of conditional vesicle accumulating intermediates. The isolation and characterization of mutants missorting vacuolar proteins (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989a; Rothman and Stevens, 1986) intended to aid further analysis of the transport of soluble vacuolar enzymes from Golgi to vacuole, assumes that a block in this particular transport event leads to the secretion of vacuolar enzymes via the default secretory pathway.

The overall half time for the migration and processing of CPY from its cytoplasmic site of biosynthesis to delivery to the vacuole has been shown to be 6 minutes (Hasilik and Tanner, 1978a). Many soluble vacuolar hydrolases move through the common compartments of the secretory pathway as inactive precursors. Only upon delivery to the vacuole do these hydrolases undergo proteolytic processing and become active. This proteolytic activation process is mediated by the product of *PEP4*,

proteinase A (PrA) (Ammerer *et al.*, 1986; Hemmings *et al.*, 1981; Jones *et al.*, 1982; Woolford *et al.*, 1986; Zubenko *et al.*, 1983). Mutants lacking active PrA often are unable to mature all other vacuolar hydrolases discovered to date (Jones *et al.*, 1982; Rupp *et al.*, 1991). Although these mutants are viable under laboratory conditions, the vacuolar proteolytic system seems to be required for survival under nutrient deprivation and the differentiation process of sporulation (Teichert *et al.*, 1989).

4.1.7. Sorting of Soluble Vacuolar Proteins

Soluble vacuolar proteins traverse the secretory pathway from the ER to the Golgi apparatus together with secretory proteins. They are selectively sorted in the late Golgi where they are separated from secretory proteins (Novick *et al.*, 1981; Klionsky *et al.*, 1990; Raymond *et al.*, 1992; Stevens *et al.*, 1982). The mechanism of this sorting process is not completely understood and differs from that of mammalian cells, because it is not dependent on protein glycosylation and phosphorylation (Schwaiger *et al.*, 1982). In certain mammalian cell lines mannose 6-phosphate serves as the secondary signal that targets certain lysosomal proteins to the lysosome (Kornfeld and Mellman, 1989). Different lines of evidence suggest that at least for CPY this is not the case, although yeast ppCPY expressed in *Xenopus* oocytes is correctly sorted to the lysosome indicative for a conserved pathway (Roitsch and Lehle, 1991). First, *S. cerevisiae* is able to efficiently sort and target CPY to the vacuole in the presence of tunicamycin. Such treatment completely blocks N-linked glycosylation in the ER (Schwaiger *et al.*, 1982; Stevens *et al.*, 1982). Second, site directed mutagenesis eliminating all four glycosylation acceptor sites present in *PRC1*, the structural gene encoding ppCPY, did not lead to a missorting of the protein product. The engineered protein product is not glycosylated, and although its transport through the secretory pathway has slower kinetics, it is still sorted to the vacuole efficiently and enzymatically active (Winther *et al.*, 1991). N-linked glycosylation may be required for the efficient sorting of PrA, or alternatively have a stabilizing function for overall stability of the normal conformation of the protein and not represent a sorting signal *per se* (Raymond *et al.*, 1992). On the

other hand, PrA-invertase hybrid proteins lacking the glycosylation sites on PrA are efficiently located to the vacuole, arguing against a role of N-linked oligosaccarides in vacuolar protein sorting (Klionsky *et al.*, 1988; Klionsky *et al.*, 1990). This hypothesis is supported by the original findings that in presence of tunicamycin CPY, PrA and aminopeptidase I are efficiently located to the vacuole (Schwaiger *et al.*, 1982).

Progress has been made in identifying the protein sequence motifs which target some representative vacuolar enzymes to their correct location (Johnson *et al.*, 1987; Klionsky *et al.*, 1988; Roberts *et al.*, 1989; Valls *et al.*, 1990). Gene fusions between varying length of the N-terminus of the vacuolar ppCPY and the secretory invertase have established that the first 50 amino acids encoded by *PRC1* (i.e. the first 30 amino acids of proCPY after signal peptide cleavage) contains information that is necessary and sufficient for targeting of the fusion protein and to the vacuole (Bankaitis *et al.*, 1986; Johnson *et al.*, 1987). Deletions and certain point mutations in the pro region of CPY led to the partial or complete secretion of CPY underlining the results obtained from the above gene fusion studies (Valls *et al.*, 1987; Valls *et al.*, 1990). The observation that the fusion of the prepropeptide of CPY to a truncated form of invertase leads to a targeting of the invertase to the vacuole confirms that the default route for soluble proteins upon entering the secretory pathway is secretion and that other targeting or retention information has to be present in proteins whose fate is not secretion (Valls *et al.*, 1987). The presence of the four contiguous amino acids QRPL in position 24 to 27 of ppCPY is required for efficient sorting of CPY (Valls *et al.*, 1990). The co-expression of CPY carrying sorting mutations within these four aminoacids and wild type CPY demonstrated that the wild type CPY was correctly sorted to the vacuole while the mutant protein was secreted (Valls *et al.*, 1990). It has been argued that these findings provide evidence that CPY is sorted as a monomer. The vacuolar PrA is also synthesized as a prepro protein, and fusion studies were able to locate the information necessary for targeting to the pro sequence (Klionsky *et al.*, 1988). However, this pro sequence has no significant identity to the CPY pro sequence, and also the complete pro sequence of PrA is required to confer targeting information. The probable location of the targeting information is within the C-terminal amino acids of the propeptide unlike

for CPY where the vacuolar targeting information is located two amino acids from the N-terminus of the propeptide (Klionsky *et al.*, 1988; Klionsky *et al.*, 1990). Little is known about the targeting information present in other soluble vacuolar proteins. It has been suggested that the propeptide of aminopeptidase I (API) contains a targeting signal (Chang and Smith, 1989; Klionsky *et al.*, 1990). The apparent difference in vacuolar targeting signals lead to the question whether there is a common sorting apparatus for all soluble vacuolar proteins or whether different vacuolar proteins are sorted by different mechanisms. Overproduction of either CPY or PrA by increasing the gene copy number of their respective structural genes results in a partial secretion of the Golgi modified form of either protein (Rothman *et al.*, 1986; Stevens *et al.*, 1986), arguing for an active, saturable receptor for vacuolar proteins in the late Golgi compartment. Overexpression of ppCPY did not result in a secretion of PrA, neither did overexpression of ppPrA result in a secretion of p2CPY. This suggests that if sorting of soluble vacuolar proteins is an active process, there are several mono- or polyspecific receptors present in a late Golgi compartment that recognize and sort different vacuolar proteins (Rothman *et al.*, 1986; Stevens *et al.*, 1986; Raymond *et al.*, 1992). An alternative explanation is that overexpressed ppCPY and ppPrA could form homo-multimers or insoluble precipitates which are no longer recognized by the sorting machinery and as a result are secreted (Raymond *et al.*, 1992). Studies in *sec1* mutants blocking transport from Golgi to the plasmamembrane show that overexpression induced secretion of p2CPY depends on transit through the late secretory pathway (Stevens *et al.*, 1986) and supports the receptor hypothesis considering that ppCPY-invertase fusions when overexpressed take the same fate (Bankaitis *et al.*, 1986). Further evidence for the presence of different sorting machineries for different soluble vacuolar proteins comes from characterization of mutants that are defective for vacuolar protein sorting (*vps*, *ssv*) and secrete a number of vacuolar proteins (Bankaitis *et al.*, 1986; Latterich and Watson, 1991; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). While most *vps* and *ssv* mutants mislocalize several vacuolar proteins, there are a few mutants which selectively secrete only one of several vacuolar enzymes tested for. A detailed analysis of one such mutant, *vps35*, provides additional

evidence that there are alternative pathways for the sorting of vacuolar proteins to the vacuole (Paravicini *et al.*, 1992). A *Δvps35* deletion mutant only mislocalizes p2CPY to the cell surface but correctly sorts PrA, PrB and API to the vacuole. It is intriguing to know that the putative gene product Vps35p lacks any signal peptide consensus and does not associate with any membraneous fraction (Paravicini *et al.*, 1992). Though not being a likely candidate for the CPY sorting receptor, it could be a part of the sorting machinery for CPY specific protein sorting.

One of the conditions required for efficient vacuolar protein sorting of soluble vacuolar proteins is vacuole acidification (Klionsky *et al.*, 1990; Klionsky *et al.*, 1992; Rothman *et al.*, 1989). Although not required for maturation of vacuolar hydrolases as suggested before (Woolford *et al.*, 1986), bafilomycin A1 induced inhibition of the vacuolar membrane ATPase is associated with a secretion of newly synthesized vacuolar proteinases p2CPY, p2PrA and proPrB. This disputes previous findings that in a *vat2* deletion mutant lacking the 60 kDa subunit of the vacuolar ATPase protein sorting is not significantly affected (Yamashiro *et al.*, 1990). Subsequent analysis of vacuolar protein sorting defects associated with disrupted genes of the vacuolar ATPase complex led to the conclusion that vacuole or vacuolar network acidification is required for the efficient sorting of soluble and membrane associated vacuolar proteins. The same sorting defects can be induced in wild type yeast cells when grown on medium whose pH is 7.5, arguing for a role of acidification of the vacuolar network (Klionsky *et al.*, 1992). The overall findings suggest that one step in the sorting machinery is sensitive to pH and requires an acidic environment to function. This could be analogous to the acidification dependent sorting receptor recycling (Mellman *et al.*, 1986).

The vacuole also derives part of its soluble constituents from the cell surface by endocytosis (Riezman, 1985), directly from the cytoplasm (Chiang and Schekman, 1991), and from the cytoplasm via peroxysomes (H.L. Chiang, personal communication). The endocytotic pathway and glucose repression associated import of fructose-1,6-bisphosphatase into vacuoles is discussed in a subsequent section (Endocytosis and Protein Degradation Pathways). All these different routes are most likely to require independent sorting or import mechanisms. Little is known about how

endocytotic vesicles release their contents to the vacuole and whether an intermediate endosomal compartment is involved (Riezman, 1985). There is also no information available of whether vacuolar proteins in hypothetical late Golgi derived vesicles share a compartment or whether the two routes are independent from each other. With the exception of Rhy1p which may be a *ras* homologue functioning in the targeting of vacuole destined vesicles to the vacuole (D. Gallwitz to M.D. Watson, personal communication) no other factors have been identified that are involved in the late transport stage.

4.1.8. Sorting of Membrane Associated Proteins

All membrane associated vacuolar enzymes appear to take the secretory pathway to transit to the vacuole (Klionsky and Emr, 1989; Klionsky *et al.*, 1990; Nishikawa *et al.*, 1990; Roberts *et al.*, 1989; Schekman, 1985) with the possible exception of α-mannosidase which enters the vacuole directly by some unknown mechanism (Yoshihisa and Anraku, 1990) and the the vacuolar ATPase subunits (except the transmembrane spanning 17 kDa subunit) which assemble in the cytosol and associate with the transmembrane subunit of the ATPase complex on the vacuolar membrane (Kane *et al.*, 1989a, 1989b). Studies of the processing pathway of the vacuolar membrane proteins repressible alkaline phosphatase (Pho8p; ALP) and dipeptidyl aminopeptidase B (DPAP B) showed that both proteins co-migrate with secretory and soluble vacuolar proteins (Klionsky and Emr, 1989; Roberts *et al.*, 1989). DPAP B is encoded by *DAP2*, a gene with a putative open reading frame of 841 amino acids. The deduced peptide has a predicted mass of 96 kDa (Roberts *et al.*, 1989). Biochemical studies showed that it is a 120 kDa integral membrane glycoprotein (Roberts *et al.*, 1989). Using *sec* mutants it was shown that DPAP B precursor forms accumulate in *sec18* and *sec7* at the restrictive temperature but not in *sec1* mutants that accumulate secretory vesicles. Immunolocalization of DPAP B in wild type and *sec* mutants confirmed that the delivery of DPAP B is blocked in *sec18* and *sec7* mutants. These observations show that DPAP B uses the same stages of the secretory pathway as

soluble vacuolar proteins. However, the co-vesicular transport of membrane bound vacuolar proteins together with soluble vacuolar proteins has not yet been investigated. Despite the fact that DPAP B and soluble proteins destined for delivery to the vacuole use the same stages, the sorting mechanism appears to be different as most *vps* mutant mislocalize soluble vacuolar proteins to the cell surface but not membrane-bound vacuolar proteins.

The other well characterized membrane associated vacuolar protein is non-specific alkaline phosphatase (ALP), a homo-dimer of 130 kDa (Onishi *et al.*, 1979), which has been shown to be identical with fructose-2,6-bisphosphate 6-phosphatase (Plankert *et al.*, 1991). A detailed biochemical analysis of the transport pathway of ALP showed that it is identical to that of DPAP B based on accumulation in *sec18* and *sec7* at the restrictive temperature (Klionsky and Emr, 1989). It also was shown that ALP, like DPAP B, is a type II integral membrane protein containing a short basic N-terminal cytoplasmic tail that is accessible to exogenous proteolytic degradation when associated both with ER and vacuole. Like DPAP B, ALP is sorted to the vacuole by a different mechanism than soluble vacuolar proteins since most *vps* mutants correctly localize ALP to the vacuole (Klionsky and Emr, 1989). ALP is the only vacuolar membrane protein known to undergo *PEP4* dependent maturation by removal of its C-terminal end (Kaneko *et al.*, 1985; Kaneko *et al.*, 1987).

Preliminary recent evidence suggests that the "default" pathway for membrane anchored proteins is delivery to the vacuole (reviewed in Raymond *et al.*, 1992). An amino acid sequence comparison between ALP and DPAP B suggests that there is no significant sequence identity. However, this is also the case for ppCPY and ppPrA and it might only be an indication of two independent sorting pathways. More compelling evidence comes from fusion studies, where a cleavable signal peptide is fused to the N-terminal lumenal domain of ALP (Klionsky and Emr, 1990). The signal peptide is cleaved, and the proteolytically processed protein which is identical to ALP is secreted, arguing that the lumenal domains do not contain sorting information. On the other hand, proteins containing the cytoplasmic domains and the transmembrane domains fused to invertase are located to the vacuole (Raymond *et al.*, 1992). Deletion of the cytoplasmic

domain of DPAP B has no effect on its vacuolar targeting, nor does replacement of the transmembrane domain with the transmembrane domain of the Golgi retained DPAP A. Both fusions are faithfully located to the vacuole arguing against targeting information present in the transmembrane domain. Also the overexpression of Golgi proteins or the deletion of the Golgi retention information present in the cytoplasmic domain of two Golgi membrane proteins DPAP A and Kex1p results in the localization to the vacuole. The fact that the overproduction of DPAP B does not result in a localization of the protein to the plasmalemma is indicative that either the default pathway is localization to the vacuole, or that the sorting pathway is not saturable (Roberts *et al.*, 1989). Up to date experimental approaches have not addressed the hypothesis that the default pathway for membrane bound proteins might still be the plasmalemma if no retention or targeting information is present, but that oversecreted or mislocalized proteins are retrieved from the plasmalemma by endocytosis. Membrane bound vacuolar proteins do not appear to proceed in this manner and normally travel from a Golgi compartment directly to the vacuole (Klionsky *et al.*, 1989; Roberts *et al.*, 1989) unlike some mammalian membrane bound lysosomal proteins which migrate to the lysosome via the plasmalemma (Mellman *et al.*, 1986). An interesting fact to remember is that overexpressed secretory transmembrane proteins are not localized to the vacuole directly from the Golgi arguing against a default pathway for membrane bound enzymes to the vacuole or saturable secretory sorting mechanism. Maybe there is no default pathway for membrane bound proteins, but their final localization depends on association with other proteins in distinct regions of the Golgi. This association could be mediated by the formation of multi-dimer lattices which rely on the interaction between certain transmembrane domains in a lipid environment as has been suggested to be involved in the Golgi retention of some proteins (G. R. Warren, open citation). It is interesting to note that all yeast transmembrane domains that result in vacuolar localization have a $G(X_{0-3})LLL$ motif 10 to 11 amino acid residues away from the N-terminal start of the transmembrane domain (M. Latterich, unpublished findings). Studies of vacuolar acidification deficient mutants suggests that vacuole acidification is required for localization of ALP to the vacuole and a collapse of the vacuolar pH

gradient leads to an accumulation of vesicles containing unprocessed ALP (Klionsky *et al.*, 1992). A similar situation is encountered when considering the differential subcellular localization of bovine β1,4-galactosyltransferase. This enzyme is synthesized by two sets of mRNA transcripts that encode two forms of the protein with a complete and extended N-terminal domains (Russo *et al.*, 1990). The truncated version is a resident type II Golgi protein while the 13 amino acid extended protein (also type II) is located to the plasma membrane (Lopez *et al.*, 1991). The conclusions drawn are that either the N-terminal extension masks a cytoplasmically located Golgi retention signal, or that it represents a positive sorting signal for localization to the plasmalemma. The differential sorting mechanism awaits elucidation.

α-mannosidase, a vacuolar inner membrane associated protein, is located to the vacuole independent to the secretory pathway (Yoshihisa and Anraku, 1989). The enzyme is a multimer consisting of 107, 73 and 31 kDa subunits the stoichiometry of which varies with the cell cycle (Yoshihisa *et al.*, 1988). The smaller subunits result from *PEP4* dependent cleavage of the 107 kDa peptide encoded by the α-mannosidase structural gene *AMS1* (Kuranda and Robbins, 1987; Yoshihisa *et al.*, 1988; Yoshihisa and Anraku, 1989; Yoshihisa and Anraku, 1990). The protein product has no obvious N-terminal signal peptide, and althogh 7 potential N-terminal glycosylation sites are present, vacuolar α-mannosidase is not glycosylated. Newly synthesized α-mannosidase is normally delivered to the vacuole and eventually matured to the 73 kDa species even when the secretory pathway is blocked by a subset of *sec* mutations. Import into the vacuole is believed to occur via a protein channel present in the vacuolar membrane. Deletion and *AMS1-SUC2* gene fusion studies indicate that the targeting information is located between amino acids 747 and 927 of the protein. The fact that the putative signal is not terminally located together with the finding that an in frame fusion of 1067 N-terminal amino acids of *AMS1* with the 511 C-terminal amino acids of *SUC2* are indicative for an internal localization signal similar to the nuclear localization signal. Import into the vacuole is a saturable event with the excess α-mannosidase activity remaining in the cytoplasm.

There is as yet no data available on the targeting of other membrane bound vacuolar enzymes to the vacuole. Out of more than 100 vacuolar membrane associated proteins only ALP, DPAP B, α-mannosidase and to some extent a 78 kDa glycoprotein (Nishikawa *et al.*, 1990) and the 17 kDa subunit of the vacuolar ATPase representing the only transmembrane component of the ATPase (Kane *et al.*, 1989a) have been characterized in terms of transport. Considering the fact that different sorting mechanisms exist for at least half of the vacuolar enzymes characterized so far it is too early to generalize on the transport phenomena. The whole picture is complicated in that different paths taken by different vacuolar constituents each of which require the presence of specific import mechanisms which also have to be localized to the vacuole. The disturbance of proper localization of any component may have secondary associated effects, as for example the missorting of proteins dependent on the vacuolar localization of the primary component.

4.1.9. Endocytosis and Protein Degradation Pathways

Most of the current research on the endocytotic pathway, representing the vesicle mediated traffic routes that interconnect the trans Golgi network, the plasmalemma, endosomes and lysosomes, has been done on mammalian cell lines (Kornfeld and Mellman, 1989) or protists (Fok and Allen, 1990). Due to the presence of a dense cell wall in *S. cerevisiae* it appears unlikely that endocytosis plays a significant role in the uptake of high molecular weight or other compounds. Fluid phase endocytosis of lucifer yellow, a sulfonate dye, has been reported to occur (Riezman, 1985). The uptake is temperature dependent and does not show saturation kinetics typical for receptor mediated endocytosis. Fluid phase endocytosis of FITC-dextran or Semliki Forest virus and vesicular stomatitis virus (Makarow and Nevalainen, 1987; Makarow, 1985a) by yeast sphaeroplasts have been elusive, since the reported uptake of FITC-dextran and accumulation of fluorescence in the vacuole was due to low molecular weight impurities present in commercial preparations of the FITC-dextran conjugate (Preston *et al.*, 1987). The use of yeast sphaeroplasts rather than intact yeast cells might not reflect

the wild type situation and caution has to be applied in interpreting endocytosis in sphaeroplasts. Although lucifer yellow shows kinetics of uptake characteristic for fluid phase endocytosis (Riezman, 1985), it has not yet been demonstrated that the dye does not enter the cell by an active process independent of endocytosis.

Evidence that yeast possesses a receptor mediated endocytic pathway comes from studies into the mechanism of mating of haploid cells. Conjugation of haploid **a** and α cells requires the action of mating pheromones before successful mating can occur (Kurjan, 1985; Sprague *et al.*, 1983). Haploid cells respond to the reciprocal mating pheromone to their own mating type. The action of α-factor on **a** cells and of **a**-factor on α-cells initiates a cellular response that finally results in forming a diploid zygote. Preliminary studies demonstrated a binding of α-factor to an α-factor receptor located on the plasmamembrane (Jenness *et al.*, 1983). The α-factor / α-factor receptor complex is internalized and delivered to the vacuole where proteolytic degradation occurs (Chvatchko *et al.*, 1986; Dulic and Riezman, 1989; Konopka *et al.*, 1988; Singer and Riezman, 1990). The same studies indicated that α-factor is internalized by a time, temperature and energy dependent process upon which it is rapidly degraded. The α-factor receptor is expressed in **a**-cells and represents a 431 amino acid integral membrane protein encoded by *STE2* (Jenness *et al.*, 1983). It has seven transmembrane domains and a C-terminal hydrophilic cytoplasmic domain. The **a**-factor receptor on α-cells shares the same structural homology with the α-factor receptor, but differs in length and seqence. The **a**-factor receptor is a 470 amino acid protein encoded by *STE3*. Both receptors are localized on the plasma membrane, and on binding of the reciprocal pheromone they are internalized and degraded in the vacuole in an *PEP4* dependent manner (Jenness and Spatrick, 1986; Raymond *et al.*, 1992). The C-terminal domains of both receptors are not involved in pheromone-induced signalling but necessary for receptor internalization (Konopka *et al.*, 1988; Raymond *et al.*, 1988; Reneke *et al.*, 1988).

Evidence for the presence of an intermediate vesicular compartment comes from kinetic and inhibitor studies. At 15°C delivery of the α-factor is slowed significantly over uptake. *PEP4* dependent degradation of the endocytosed α-factor is not rate

limiting at 15°C, and there is biochemical evidence of the accumulation of a transport intermediate. A protease protected transport intermediate can also be isolated after NaN_3 or NaF treatment which is distinct from plasma membrane and vacuole as judged by differential and density gradient centrifugation. Whether this transport intermediate represents a vesicular intermediate in plasma membrane to Golgi transport, an endosome-like compartment or both is not yet clear.

How the ligand-receptor complexes are internalized is not known. Clathrin, which is required for receptor mediated endocytosis in mammals (Goldstein *et al.*, 1985), is not required for α-factor uptake in *S. cerevisiae* (Payne *et al.*, 1988). Although clathrin heavy chain mutants display a reduced rate of α-factor uptake compared to wild type cells, this might simply be due to the reduced growth rate of the mutant. There is evidence that some of the *SEC* genes involved in transport in the secretory pathway (Novick *et al.*, 1980; Novick *et al.*, 1981) also are involved in endocytosis (Riezman, 1985). However, these results were based on fluid phase endocytosis of lucifer yellow, whose uptake may not reflect true endocytosis (Raymond *et al.*, 1992). It is not known how significant endocytosis is in vacuole biogenesis, and whether pinocytosis occurs. It would be interesting to see if *S. cerevisiae* endocytoses nutrients for degradation in the vacuole and how significant this hypothetical event is in survival under unfavourable conditions. It is also not known whether endocytotic membrane flow significantly contributes to the vacuolar membrane. Is there a recycling pathway that will recycle endocytotic membrane vesicles back to the plasma membrane, maybe via an endosome? The answer is not clear, but there appears to be no need for mating factor receptor recycling. The directed budding, targeting and fusion of endocytic vesicles with the receptor compartment(s) will require factors specific for that particular transport step. The identification of such factors and the design of an *in vitro* system will help in an understanding of this pathway. An *in vitro* system that allows the cell-free reconstitution of transport from the late endosome to the lysososme in mammalian cells established transport dependence on ATP and cytosol (Mullock *et al.*, 1989). It should be interesting to see whether yeast cytosol can substitute the cytosolic factors involved in this pathway.

Protein degradation of cytoplasmic proteins in the vacuole has been suggested before (Klar and Halvorson, 1975), but it was not until much later that the selective import of a protein destined for vacuolar degradation has been described (Chiang and Schekman, 1991; Raymond *et al.*, 1992). Indirect evidence points towards degradation of Golgi localized Kex1p, Kex2p and DPAP A in the vacuole (Raymond *et al.* 1992). In case of Kex2p the half life of the protein is extended in *pep4* mutants compared to the isogenic wild type, but whether this phenomena is due to *PEP4* dependent degradation in the vacuole is not known.

Fructose 1,6-bisphosphatase (FBPase), a key regulatory enzyme in gluconeogenesis, is synthesized when yeasts are grown on a poor carbon source such as ethanol. Upon transfer to glucose containing growth medium, FBPase synthesis is repressed and the cytoplasmic enzyme is phosphorylated and degraded (Guerra *et al.* 1988; Schafer *et al.*, 1987). The degradation is dependent on the *PEP4* gene product and comparatative immunoprecipitation studies led to the conclusion that the degradation of FBPase is a direct response to glucose (Chiang and Schekman, 1991). Subcellular fractionation and indirect immunofluorescence using an anti-FBPase antibody showed that the FBPase was taken up by the vacuole upon transfer of a *pep4* mutant from a poor carbon source to glucose. It appears likely that the FBPase destined for degradation enters the vacuole or a post-ER compartment beacause the three potential glycosylation sites on the protein are not glycosylated and the protein lacks an obvious signal peptide for translocation into the ER. It is not yet known how the protein is translocated across the vacuolar membrane or a prevacuolar compartment, but *PEP4 sec* mutant strains blocking transport at different stages did retard the catabolite repression induced degradation of FBPase in *PEP4 sec62*, *PEP4 sec18*, *PEP4 sec7* but not *PEP4 sec1* at the restrictive temperature. This might be characteristic for the glucose induced expression of a protein that traverses the secretory pathway to the vacuole and is required for uptake of FBPase. The fact that in the presence of cycloheximide FBPase uptake into the vacuole does not occur corroborates these results. The translocation of FBPase into a membranous compartment occurs at a post ER stage since *sec18* cells do not import FBPase into the ER during catabolite

repression. Together with the fact that α-mannosidase is targeted into the vacuole directly, bypassing the secretory pathway (Yoshihisa and Anraku, 1990), there appears to be a mechanism for regulated uptake and degradation of specific cytoplasmic compounds under certain metabolic conditions. Mammalian cells and yeast have different bulk degradation pathway for the rapid turn-over of proteins in the lysosome or vacuole, respectively (Chiang *et al.*, 1989; Dice, 1990; Teichert *et al.*, 1989; Zubenko and Jones, 1981). Hepatocytes deprived of amino acids will enclose random cytoplasmic material in ER derived autophagic vacuoles which will fuse with or "become" lysosomes (Dunn, 1990a; Dunn, 1990b). Little is known whether bulk degradation in *S. cerevisiae* occurs analogously or whether different mechanisms operate to ensure bulk delivery to the vacuole. The engulfing of cytoplasmic material by vacuoles has been observed in yeast (Vorisek, 1989), but since the findings are solely based on observation of electron micrographs, caution has to be applied not to overinterpret potentially artefactual appearances. Selective import of proteins into the lysosome in serum deprived cells requires the presence of a peptide motif within the proteins to be degraded (Dice, 1990). The motif is KFERQ, or related sequences with the same chemical properties, which is recognized by a 73 kDa peptide recognition protein (73prp) which is identical to hsp70 binding to and disassembling clathrin light chains. Interestingly, clathrin light chain has two overlapping peptides related to the KFERQ motif (Chappell *et al.*, 1986; Dice, 1990). Similar motifs have been suggested to mediate the import of F6BPase into the yeast vacuole (Chiang and Schekman, 1991).

The proteolytic activity of the vacuole is associated with general protein degradation as part of the sporulation response (Achstetter and Wolf, 1985; Heinemeyer *et al.*, 1991). Inactivation of the *PEP4* gene product leads to a drastic reduction in sporulation efficiency due to the pleiotropic inactivation of proteinases that depend on PrA activation. These proteinases function in the rapid turnover of unecessary cellular proteins during sporulation to provide amino acids for new sporulation specific gene products. The sporulation response can occur in the absence of an exogenous nitrogen source, and about 30% of vegetative proteins are turned over during sporulation (Klar and Halvorson, 1975). PrA and PrB are responsible for the majority of non-specific

protein degradation under nutritional stress conditions (Teichert *et al.*, 1989). The import of proteins destined for degradation into the vacuole is at present subject to speculation. There is evidence that the yeast vacuole participates in the degradation of ubiquinated proteins also (Heinemeyer *et al.*, 1991). Considering the fact that proteinase yscE represents the yeast proteasome (Achstetter *et al.*, 1984; Kleinschmidt *et al.*, 1988) required for the degradation of ubiquitin tagged proteins (Heinemeyer *et al.*, 1991), and that mutants in subunits of the proteosome are lethal, it is interesting that there is a second pathway of degrading ubiquitin labelled proteins. Maybe there is a functional specialization between proteosome mediated degradation of regulatory proteins and (maybe) general turnover of ubiquinated proteins in the vacuole or mediated by a vacuolar process.

4.1.10. Biosynthesis of Soluble Vacuolar Proteins

Aminopeptidase Co. Aminopeptidase Co (APC) is a soluble metallo-exopeptidase with a molecular mass of approximately 100 kDa. Its activity depends on the presence of Co^{2+} ions, and is inhibited in the presence of Zn^{2+} and EDTA (Achstetter *et al.*, 1982). Its activity increases when cells reach stationary growth phase (Achstetter *et al.*, 1984), similar to other vacuolar proteinases. There is currently no available information about its structural gene and its processing pathway.

Aminopeptidase I. Aminopeptidase I (API) is a multimeric soluble metallo-exopeptidase of a molecular mass of about 640 kDa (Frey and Röhm, 1978). It is activated in the presence of Zn^{2+} (Metz and Röhm, 1976), and is composed of 12 identical glycoprotein subunits of approximately 50 to 53 kDa (Metz and Röhm, 1976; Klionsky *et al.*, 1990). API activation is *PEP4* dependent (Jones, 1991). Its structural gene, LAP4, was cloned and sequenced (Chang and Smith, 1989; Cueva *et al.*, 1989). It encodes a 514 amino acid protein with a 45 amino acid prosequence at the N-terminus. API is synthesized as a 57 kDa precursor containing four potential glycosylation sites of which two seem to be utilized. The extreme N-terminus of the protein does not have a signal peptide conforming to von Heijne's rules (von Heijne, 1983). Part of the pro-

sequence does have a hydrophobic/hydrophilic stretch that can be arranged into an ampiphilic α-helix and could represent a translocation signal peptide. The enzyme has a pH optimum in the neutral to basic range (Frey and Röhm, 1978).

Carboxypeptidase S. Carboxypeptidase S (CPS) has been identified in mutants deficient in PrA, PrB and CPY activities (Wolf and Ehmann, 1978). It is encoded by *CPS1*, and little is known about its biosynthesis (Jones *et al.*, 1982). CPS is constitutively active and thought to supply cells with amino acids when external amino acids or a nitrogen source is not available. Its activity is dependent on the presence of Zn^{2+} and is regulated by the available nitrogen source (Achstetter and Wolf, 1985).

Carboxypeptidase Y. Carboxypeptidase Y (CPY) is a soluble serine peptidase representing a monomeric glycoprotein with a molecular mass of approximately 61 kDa (Achstetter and Wolf, 1985). CPY is the most extensively characterized vacuolar enzyme, and many of the pioneer studies that established the pathway of transit to the vacuole as well as vacuolar sorting relied upon the study of the biogenesis of CPY (see 3.1; 4.1.6.). In the following only some general features of the enzyme will be discussed. ppCPY is encoded by *PRC1* (Wolf and Fink, 1975) which has been cloned by its overexpression induced secretory defect (Stevens *et al.*, 1986). The gene encodes a 532 amino acid protein with no significant homology at the amino acid level with other vacuolar proteinases. The first 20 amino acids represent an ER specific signal peptide. Amino acids 19 to 111 represent the pro-peptide which is cleaved in a *PEP4* and *PRB1* dependent manner (Mechler *et al.*, 1987). Removal of the signal peptide by deletion of amino acids 2 to 28 did not abolish CPY's ability to translocate into the ER but merely slowed its kinetics. The Δ2-28CPY that entered the ER was secreted into the medium (Blachly-Dyson and Stevens, 1987). This led to the idea that the first few amino acids of the pro-peptide contained vacuolar targeting information. Two approaches using deletions in the pro-sequence of ppCPY (Valls *et al.*, 1987; Valls *et al.*, 1990) and gene fusions of differing lengths of the N-terminus of CPY with invertase (Johnson *et al.*, 1987) demonstrate that the first 30 amino acids of the propeptide are necessary and essential for vacuole delivery. Site directed mutagenesis identified that Gln-24 to Leu-27 form a vacuolar targeting signal. The cis-dominance of such

mutations are also indicative of interaction with a sorting apparatus conferring specificity rather than an unspecific sorting event. ppCPY undergoes N-linked glycosylation and is proteolytically processed in several events (Klionsky *et al.*, 1990). The pro-peptide is important in maintaining CPY inactive until delivery to the vacuole (Achstetter and Wolf, 1985; Hasilik and Tanner, 1978a), a feature which has been observed with some other vacuolar enzyme precursors such as PrA, PrB, API (Jones, 1991), trehalase (Har2ris and Cotter, 1987) and RNAse (Jones *et al.*, 1982). Most of the activation is mediated by PrA and PrB (Jones, 1991). The pro-peptide of CPY has also been associated with the stabilising of the protein conformation and the correct self-assembly during translocation and migration through the secretory pathway (Winther and Sørensen, 1991). Like other proteases, the activity of CPY is regulated and activity of CPY increases several-fold when cells enter diauxic growth, stationary phase or under starvation conditions (Distel *et al.*, 1983; Hansen *et al.*, 1977; Klar and Halvorson, 1975; Saheki and Holzer, 1975). The levels of CPY mRNA increase up to ten-fold at the end of the exponential growth period on glucose as a carbon source (Distel *et al.*, 1983). A 6- to 8-fold increase in the gene copy number of *PRC1* leads to a 50 to 55% secretion of the p2CPY synthesized during the exponential growth phase (Stevens *et al.*, 1986). It has not been examined whether secretion of the p2CPY form naturally occurs during the life cycle and has a biological function in degradation of peptides present in the extracellular environment, especially since secreted p2CPY undergoes extracellular activation (Nielsen *et al.*, 1990; Rothman and Stevens, 1986). This hypothetical phenomenon would be consistent with the observation that CPY expression is induced when cells grow on a peptide which is a substrate for the enzyme (Achstetter and Wolf, 1985).

Phosphatases (Non-membrane Asscociated). There are two polyphophatases, endo-polyphosphatase and exo-phosphatase, highly enriched in vacuole preparations (Dürr *et al.*, 1979; Wiemken *et al.*, 1979). So far no data are available about their function and biogenesis.

Proteinase A. Proteinase A (PrA) is a soluble, monomeric, carboxylic endopeptidase (Achstetter and Wolf, 1985). It is encoded by *PEP4* which has been

cloned by overexpression induced secretion in an immunological screen (Rothman *et al.*, 1986). *PEP4* encodes a 405 amino acid precursor (ppPrA) with significant homology to the mammalian aspartyl proteases pepsin, renin and cathepsin D (Ammerer *et al.*, 1986). The extreme N-terminus of ppPrA constitutes a signal peptide which is cleaved upon entry into the ER between amino acids 22 and 23 (Klionsky *et al.*, 1988). The protein is core glycosylated on two sites corresponding to amino acid 144 and amino acid 345 (p1PrA) migrating on SDS-PAGE with a molecular mass of 48 kDa (Ammerer *et al.*, 1986). The protein has four cysteines which are believed to be engaged in the formation of disulphide bonds (Dreyer *et al.*, 1986). During transit through the ER and Golgi p1PrA undergoes core oligosaccharide trimming and outer chain modification to yield p2PrA (52 kDa). The propeptide is cleaved between Glu-76 and Gly-77 of the predicted protein sequence to form the mature active 42 kDa PrA upon delivery to the vacuole (Ammerer *et al.*, 1986) in what is believed to be an autocatalytic process (Mechler *et al.*, 1987). Site directed mutagenesis of the active catalytic site of PrA, exchanging Asp-294 with Ala-294, led to an inactivation of catalytic activity of the enzyme (Rupp *et al.*, 1991). Cells expressing such a mutated PrA failed to proteolytically process the mutant PrA, supporting the view that p2PrA undergoes autocatalytic activation. The propeptide contains vacuolar targeting information (Klionsky *et al.*, 1988), but a consensus sequence has not been established yet (see above). PrA has a preference for Phe, Leu and Glu in the -1 position and for Phe, Ile, Leu and Ala in the 1 position of the cleavage site (Dreyer, 1989). Its substrate specificity is more limited than for example the related pepsin, and its cleavage pattern reflects that of cathepsin D, except that PrA is not restricted to cleavage between large hydrophobic amino acids. PrA dependent maturation of carboxypeptidase Y, proteinase B and autocatalysis is not solely accountable for the proteolytic action of PrA since its specificity does not account for cleavage at the cleavage points (see above). Rather the synergistic action of PrA and PrB is accountable for the observed processing of PrA, PrB and CPY (Jones, 1991). After autocatalytic removal of 47 N-terminal amino acids of p2PrA immediately before or upon delivery to the vacuole the remaining 7 amino acids of the propeptide are removed in a PrB catalyzed reaction. PrA activity is

regulated, and the PrA activity increases several-fold when cells enter stationary or diauxic growth phase (Hansen *et al.*, 1977) or as a sporulation specific response (Klar and Halvorson, 1975). PrA as well as PrB play an essential role in the maturation of proteinases and the starvation induced vegetative protein degradation (Jones, 1991; Teichert *et al.*, 1989). PrA migrates through the secretory pathway to the vacuole with a half-time of 6 minutes which is identical to that of CPY, suggesting that it reaches the vacuole via the same compartmental route or mechanism (Klionsky *et al.*, 1990).

Proteinase B. Proteinase B is a soluble serine-, sulphydryl endopeptidase with a pH optimum for proteolysis in the neutral range (Achstetter and Wolf, 1985). It is a monomeric enzyme with a molecular weight of 33 kDa, of which 10% represents carbohydrate insensitive to Endo H treatment. This is characteristic for an O-linked carbohydrate moiety. Its structural gene, *PRB1*, has been cloned and sequenced (Moehle *et al.*, 1987). It encodes an approximately 70 kDa protein, of which the first 19 residues have the characteristics of a signal peptide supporting the view that PrB enters the secretory pathway at the ER level. Using specific antisera combined with pulse chase and genetic experiments its maturation pathway has been established (Hirsch *et al.*, 1992; Moehle *et al.*, 1989). Upon translocation of the ppPrB protein into the ER the signal peptide is cleaved by the signal peptidase and the protein receives one asparagine linked and an unknown number of O-linked carbohydrate side chains. The glycosylated intermediate is proteolytically processed to a 39 kDa form while in the ER (p1PrB), which becomes 40 kDa in the Golgi due to outer chain modification of the Asn-linked side chain (p2PrB). Upon arrival in the vacuole the p2PrB is converted to the mature 31 kDa form via a 37 kDa intermediate. Site directed mutagenesis, substituting the catalytical Ser-519 of the active site with Ala-519 led to the formation of an inactive protein that enters the secretory pathway and reaches the vacuole as a 73 kDa protein but is not processed (Hirsch *et al.*, 1992; Nebes and Jones, 1991). The active site mutation thus prevents clevage of the 41 kDa peptide called superpeptide and the subsequent cleavage of the C-terminal propeptide. Evidence suggests that in wild type cells the superpeptide remains associated with the pPrB until the vacuolar compartment is reached. In the vacuole PrA dependent cleavage of most of the propeptide takes place

resulting in the activation of PrB. The final trimming of the remaining amino acids of the propeptide, as well as the rapid degradation of the superpeptide is belived to occur as part of an autocatalytic event (Hirsch *et al.*, 1992; Jones, 1991). At present unknown is the location of the vacuolar targeting signal, which might be located in the C-terminal propeptide or the superpeptide that remains associated with the pPrB (Klionsky *et al.*, 1990). PrB levels, like PrA activity, are subject to environmental regulation (Klar and Halvorson, 1975; Moehle and Jones, 1990). *PRB1* transcription is repressed in the presence of glucose, and also subject to changes in the life cycle because *PRB1* transcription is induced as cells reach the stationary growth phase. Transcription is tightly regulated since an increase in copy number of the structural gene does not result in an overproduction of PrB under unfavourable glucose conditions. It has been suggested that *SNF2* and *SNF5*, two of at least six genes required for the derepression of secreted invertase synthesis (Carlson *et al.*, 1981) are transcriptional regulators of *PRB1*. PrB appears to be required for effective sporulation, proteolytic processes during nitrogen starvation and for the "trimming" of PrA cleaved vacuolar hydrolases (Jones, 1991). PrB is specifically expressed after irradiation with UV and enzyme activity is dose-dependent, althogh not much is known about the regulatory mechanism (Schwencke and Moustacchi, 1982). The transport and processing occurs with similar efficiency to that of CPY, suggesting that PrB as well as PrA and CPY share the same vesicular transport pathway (Moehle *et al.*, 1989).

Ribonucleases. Ribonuclease activity has been found in the vacuole (Wiemken *et al.*, 1979) but nothing is known about its biosynthesis or biological function. The presence or import of RNA, its natural substrate, has not yet been shown. Ribonucleases in mammalian cells are selectively imported and degraded in lysosomes (Dice, 1990). This could also be the case in *S. cerevisiae*, and the activity found in vacuoles might be residual activity of the RNAses before inactivation.

Trehalase. Trehalase is the sole enzyme involved in the catabolism of trehalose, a storage carbohydrate (Thevelein, 1984) and stress protectant (Hottiger *et al.*, 1987). There are two forms of trehalase in *S. cerevisiae*, a cAMP dependent cytosolic enzyme and a vacuolar enzyme (Keller *et al.*, 1982; Thevelein, 1984). The cytosolic enzyme is

tightly regulated and only active when phosphorylated. The vacuolar enzyme is a glycoprotein of 215 kDa (Londesborough and Varimo, 1984) and transits through the early secretory pathway to the vacuole (Harris and Cotter, 1988). Its activation is *PEP4* dependent (Harris and Cotter, 1987). The biological function of the vacuolar enzyme is unknown and not essential under physiological conditions.

4.1.11. Biogenesis of Vacuolar Membrane Associated Proteins

α-mannosidase. α-mannosidase is an inner peripheral or ecto- vacuolar membrane protein that is associated with the membrane other than by a membrane spanning domain. The enzyme represents a 560 kDa multimer consisting of 107, 73 and 31 kDa subunits (Yoshihisa *et al.*, 1988). It appears likely that the 73 and 31 kDa subunits are due to specific proteolysis, since both are antigenically related to the 107 kDa species and occur in an equimolar ratio. This proteolytic event occurs prevalently in the stationary phase. The structural gene for α-mannosidase, *AMS1*, has been cloned and sequenced (Kuranda and Robbins, 1987; Yoshihisa and Anraku, 1989). It encodes a 107 kDa polypeptide with no obvious signal sequence and seven N-glycosylation consensus sites. A deletion mutant of the *AMS1* gene does not synthesize the 73 and 31 kDa fragments, representing indirect evidence that they are derived from the 107 kDa fragment. α-mannosidase enters the vacuolar sytem directly bypassing the secretory pathway (Yoshihisa and Anraku, 1990), although the exact mechanism for translocation is unknown. α-mannosidase is subject to carbon catabolite repression and sporulation induced increase in activity, although its biological function is not known (Opheim, 1978). It appears unlikely that α-mannosidase and fructose 1,6-bisphosphatase, both of which are selectively sequestered in the vacuole under certain conditions, take the same specific import pathway, because the uptake of FBPase depends on the expression of a, probably, vacuolar protein that transits the secretory pathway before uptake can occur (Chiang and Schekman, 1991). The uptake of α-mannosidase depends on the presence of a most likely constitutively expressed transporter (Yoshihisa and Anraku, 1990). The biological significance of this vacuolar import pathway has been disputed because of its

slow half-time of import, and it has been suggested that its uptake is part of a degradation process (Preston *et al.*, 1991). On the other hand, unmasked α-mannosidase activity is found predominantly in the vacuole which has led to its use as vacuolar marker enzyme (Opheim, 1978)

Alkaline Phosphatase. Among several phophatases located to the vacuole is one membrane associated alkaline phosphatase which is regulated in response to phosphate levels involving a regulatory system including Pho4p, Pho80p, Pho81p and Pho85p (Oshima, 1982). It has a molecular mass of 130 kDa and is a glycosylated homodimer (Onishi *et al.*, 1979). Its structural gene, *PHO8*, has been cloned and sequenced (Kaneko *et al.*, 1985; Kaneko *et al.*, 1987). It encodes a 566 amino acid protein with one hydrophobic region near the N-terminus and two N-linked glycosylation sites in position 268 and 401. The protein is glycosylated and transits to the vacuole where *PEP4* dependent proteolytic removal of a C-terminal peptide occurs. The half-time of transport is 6 minutes, suggesting that ALP shares the same transport pathway as soluble vacuolar enzymes. Protease protection studies confirmed that ALP is a type II membrane protein. The sorting signal of ALP is unknown, in fact it has been suggested that sorting of vacuolar membrane proteins occurs by default without any sorting information (Raymond *et al.*, 1992). The role of this non-specific enzyme is unclear. An insight comes from the recent discovery that fructose 2,6-bisphosphate 6-phosphatase (Fru2,6P_2-ase) is encoded by *PHO8*, confirming the indentity of both enzymes. Fru2,6P_2 plays an important role in the regulation of overall carbohydrate metabolism. Fru2,6P_2 is rapidly formed as a response to high glucose levels which is believed to be a secondary effect resulting from an increase of cAMP levels (Hers *et al.*, 1988). It represents a low molecular weight stimulator of 6-phosphofructo-1-kinase and simultaneously an inhibitor of fructose-1,6-bisphosphatase, and thus acts as a key regulatory metabolite in regulating glycolysis and gluconeogenesis. Upon shifting yeast cells to glucose containing growth medium the consequent increase of cAMP will activate protein kinase which subsequently phosphorylates at least three proteins: trehalase (activated), 6-phosphofructo-2-kinase (activated) and fructose-1,6-bisphosphatase (inactivated). Fru2,6P_2 is degraded by fructose-2,6-bisphosphate 6-

phosphatase, presumably in the absence of glucose. The question remains why this enzyme is located to the vacuolar membrane and why it is activated upon delivery to the vacuole and not before. The vacuole might participate in regulating carbohydrate metabolism in yeast, maybe by importing fructose-2,6-bisphosphate for degradation. This would be analogous to import of fructose-1,6-bisphosphatase for degradation under glucose repression (Chiang and Schekman, 1991).

AMPase. The presence of a vacuolar membrane bound AMPase has been suggested by a number of studies (Schwencke, 1977; Wiemken *et al.*, 1979) although no further data are available yet.

ATPase. The vacuoler membrane H^+-ATPase is a complex consisting of at least three subunits (Arai *et al.*, 1988; Bowman *et al.*, 1986; Kane *et al.*, 1989a; Kane *et al.*, 1989b; Mandala and Taiz, 1985; Uchida *et al.*, 1985). It plays a crucial role in the acidification of the vacuole or the vacuolar network (Mellman *et al.*, 1986) and thus is required for a number of vacuolar transport processes including the transport of ions and amino acids (Ohsumi and Anraku, 1983; Ohsumi *et al.*, 1988; Okorokov *et al.*, 1985; Sato *et al.*, 1984a) and the efficient sorting of soluble and membrane bound vacoular proteins (Klionsky *et al.*, 1992). Its role in providing an acid environment for proteolysis has been suggested because the pH optimum of a number of vacuolar hydrolases is in the slightly acidic range (Woolford *et al.*, 1986), but a recent investigation showed that it is not required for efficient proteolysis to occur (Klionsky *et al.*, 1992). The vacuolar H^+-ATPase is distinct from other F_1F_0 ATPases including *E. coli*, mitochondrial and chloroplastic ATPases and also from the plasma membrane E_1E_2 ATPases in terms of immunological crossreactivity (Bowman and Bowman, 1986) and inhibitor spectra (Bowman and Bowman, 1986; Bowman *et al.*, 1988b). It is sensitive to bafilomycin A_1, N-ethylmaleimide and cynanide, and insensitive to azide and vanadate. The H^+-ATPase complex contains three major subunits of 69, 60 and 17 kDa and other proteins of 100, 42, 36, 32 and 27 kDa in association (Kane *et al.*, 1989b). The same study was able to show that the 69 kDa subunit contains the catalytic site, mediating ATP binding alone or in conjunction with other subunits. These subunits bind radioactive ATP analogues such as 8-azido-ATP and are labeled by ATPase

inhibitos such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and N-ethylmaleimide (Uchida *et al.*, 1988). It has been proposed that the 60 kDa subunit represents a regulatory ATP-binding subunit and the 17 kDa subunit the proton pore (Kane *et al.*, 1989a). Another proteolipid, the 16 kDa subunit, has been found which is distinct from the 17 kDa proteolipid (Umemoto *et al.*, 1991). Little is known about the assembly of the ATPase, but it has been suggested that the ATP binding subunits are located on the cytoplasmic face of the vacuolar membrane based on the fact that the putative catalytic subunit (69 kDa) can be stripped off from vacuolar vesicles (Ohsumi and Anraku, 1981). Substrate accessibility is also restricted to the cytoplasmic site of the membrane (Kakinuma *et al.*, 1981), which is inferred from the function of the ATPase in acidifying the interior (Klionsky *et al.*, 1990). The other subunits have not been assigned a function yet, but it is believed that they either play a role in assembly or mediate attachment of the 69 and 60 kDa subunits to the 17 kDa pore complex (Kane *et al.*, 1989a). They have subsequently been shown to be essential for vacuole acidification since disruptions of the structural genes encoding the 70, 60, 42, 27, 17 and 16 kDa subunit lead to a complete inactivation of the ATPase *in vitro* and *in vivo* (Raymond *et al.*, 1992). The same effect can be observed by cold KNO_3 inactivation, where the majority of subunits are released (Kane *et al.*, 1989b). It has been suggested that the 17 kDa subunit migrates through the secretory pathway as a transmembrane protein, and the actual assembly of the other cytoplasmic subunits take place on the vacuole or a prevacuolar compartment, but conclusive evidence is still missing. Several approaches have been taken to elaborate the mechanisms of its assembly and function. Kinetic studies revealed that the process of catalysis is similar to that of F_0F_1 ATPases in that it requires cooperativity between subunits and several ATP molecules per enzyme (Hirata *et al.*, 1989; Kasho and Boyer, 1989; Uchida *et al.*, 1988). Unlike F_0F_1 ATPases where the disassembled F_1 subunit complex can carry out ATP hydrolysis independently, none of the vacuolar ATPase subunits retaines the ability to hydrolyse ATP (Kane *et al.*, 1989b). Several approaches have been taken to isolate mutations in the structural genes of the subunits of the vacuolar ATPase. Based on the fact that acidification of the vacuole is dependent on the assembly of all subunits into a complex

and that vacuolar proteins are missorted, the examination of mutants defective in vacuolar protein localization yielded two mutants defective in vacuole acidification as judged by their failure to accumulate quinacrine (Preston *et al.*, 1989; Rothman *et al.*, 1989c). Two direct approaches were taken to generate mutants in vacuole acidification and the ATPase. One method selected for mutants with reduced fluorescence after labeling with 6-carboxyfluorescein. One recessive mutant, *vph1*-1, was identified that maintains the vacuolar pH at 6.9 (Preston *et al.*, 1989). The other approach concentrates on 2cloning the genes corresponding to the vacuolar ATPase subunits. The following genes corresponding to the ATPase subunits have been cloned, *VMA1* (70 kDa), *VMA2*, *VAT2* (60 kDa), *VMA3* (16 kDa), *VMA4* (27 kDa), *VMA5* (42 kDa) and *VMA6* (36 kDa) (Anraku *et al.*, 1989; Klionsky *et al.*, 1990; Raymond *et al.*, 1992; Rothman *et al.*, 1989). *VMA1* was cloned by hybridisation probing of a yeast genomic DNA library with a degenerate oligonucleotide probe whose sequence was deduced from the amino acid sequence of a protein fragment (Anraku *et al.*, 1989; Hirata *et al.*, 1989). *VMA1* encodes a 1071 amino acid protein, whose 285 N-terminal and 332 C-terminal amino acids are 75% identical with the catalytic subunit of the *Neurospora crassa* H^+-ATPase (Bowman *et al.*, 1988). The 454 amino acid central domain is not essential to the ATPase and is believed to be removed by a protein processing mechanism or by autocatalysis to yield a 67 kDa protein (Anraku *et al.*, 1989). It would be tempting to speculate that this processing event could be involved in activation or assembly of the ATPase. A disruption of *VMA1* results in low spore viability and a reduced growth rate but is not essential for vegetative growth. *VAT2* was cloned by hybridisation probing of a yeast genomic library with a degenerate oligonucleotide whose sequence was deduced from the amino-terminal protein sequence (Yamashiro *et al.*, 1990). It encodes a 517 amino acid protein with a predicted molcular mass of 58 kDa without an obvious ER-signal sequence. The protein was identical to the 57 kDa H^+-ATPase isolated before (Nelson *et al.*, 1989), except the C-terminus which was probably due to a sequencing artefact of the first sequence established. A deletion of *VAT2* did not result in lethality but rather conferred an acidification defective phenotype, inability to grow on neutral growth medium and a weak vacuolar protein sorting defect (Yamashiro *et al.*, 1990).

Zymogen activation was not impeded in the mutant. Other vacuole associated functions like amino acid storage were not examined in the deletion mutant. Its doubling time under normal growth conditions is significantly greater than the wild type. *VMA3* was also cloned in a library screen with a degenerate oligonucleotide (Umemoto *et al.*, 1990). The gene has the potential to encode a 160 amino acid protein, which shares extensive sequence homology (65%) with the 16 kDa subunit of the chromaffin granule ATPase (Mandel *et al.*, 1988). A disruption of the *VMA3* gene has a similar growth defect as Δ*vma1* and Δ*vat2*. *VMA4* is the structural gene for the 27 kDa subunit of the vacuolar ATPase. Its encodes a 233 amino acid hydrophilic polypeptide which exhibit 34% sequence identity with the E subunit of the lysosomal ATPase from bovine microsomes (Foury, 1990). Judging from a deletion mutant the protein is not essential for vegetative growth, but it is defective in vacuolar ATPase activity and is cold- and temperature-sensitive. The structural genes for the 42 and 36 kDa subunits, *VMA5* and *VMA6* respectively, have been cloned and sequenced (Beltrán *et al.*, 1992; Raymond *et al.*, 1992). They show homology to subunits of similar size from *Neurospora crassa* and bovine ATPase, and they are not essential under physiological conditions. The 42 kDa subunit (Vma5p; C-subunit) function can be rescued by the bovine equivalent of the subunit in a gene fusion between the yeast vcuolar ATPase and bovine V-ATPase, and it is essential for the assembly of the catalytic center of the enzyme (Beltrán *et al.*, 1992). Other genes have been identified which participate in vacuolar ATPase assembly or activation (Raymond *et al.*, 1992). *VMA12* and *VMA13* mutants are deficient in the assembly of vacuolar ATPase, yet the genes do not encode abundant subunits of the ATPase complex (Ohya *et al.*, 1991). Other vacuole acidification deficient mutants, *vph1*, *vps3* and *vps6*, exhibit ATPase assembly defects although none of the gene products encode subunits of the ATPase (Raymond *et al.*, 1992; Rothman *et al.*, 1989b). Some 8 additional *vps* mutant strains fail to assemble the ATPase complex. It is difficult to judge at present whether the protein sorting defect results in a mislocalization of vacuolar proteins important for ATPase assembly, or whether the acidification defect leads to a missorting of vacuolar proteins. Lastly disruptions in genes corresponding to subunits A (70 kDa), B (60 kDa) and c (16 kDa) of the ATPase confer a *vps*-phenotype

(Klionsky *et al.*, 1992). It is difficult to judge at present which vacuolar and non-vacuolar protein constituents are primary participants in assembly and function of the vacuole and which affect other vacuolar functions by disturbing the regulated equilibrium subject to control at many levels.

Mutational analysis of a number of biochemical pathways has successfully been used to identify protein constituents participating in the biochemical event or involved in the regulation and gene expression of such constituents. Biochemical pathways sometimes are less complex than the biogenesis of a vacuole, and individual steps can be analysed in a cell free system. On the other hand, the secretory pathway has successfully been analysed by generating conditional mutants, but in case of a mutation inactivating an essential protein would be lethal. The vacuole is not an essential organelle, and a mutation in an essential protein constituent will not be lethal under physiological conditions. The reconstitution of the vacuole biogenesis pathway is difficult because of the sensitivity of the vacuole during isolation. The yeast plasma membrane H^+-ATPase, which is localized via transit through the secretory pathway, is activated by progressive compartment dependent specific phosphorylation on multiple Ser and Thr residues during transit (Chang and Slayman, 1991). Upon arrival at the plasma membrane it undergoes specific phosphorylation of specific sites which regulate its activity in response to glucose. Whether the ATPase is active between assembly on the ER membrane and the exit of the Golgi is unknown, but a similar mechanism for the vacuolar ATPase could exist to regulate its activity by progressive phosphorylation.

Dipeptidy aminopeptidase B. Dipeptidyl aminopeptidase B (Dipeptidyl aminopeptidase V; DPAP B) is a membrane associated exopeptidase (Bordallo *et al.*, 1984). Its structural gene, *DAP2*, has been cloned as a multicopy suppressor of a *ste13* mutant which lacks the Golgi dipeptidyl aminopeptidase A (DPAP A). Disruption of the locus led to an abolishment in DPAP B activity (Roberts *et al.*, 1989) similar to mutants deficient in DPAP B activity (Suárez Rendueles and Wolf, 1987). *DAP2* encodes a 841 amino acid protein of a predicted molecular mass of 96 kDa and with a single hydrophobic domain near the N-terminus. The predicted sequence possesses 8 potential N-glycosylation sites of which at least 5 are used. DPAP B has a molecular mass of 110

to 113 kDa upon arrival in the vacuole and is not removed from the vacuole membrane by Na_2CO_3 treatment. This plus presence of only one hydrophobic domain indicates that DPAP B has the characteristic of a type II transmembrane protein. The Golgi and vacuole species do not differ in size, indicating that proteolytic processing does not occur. This is consistent with the finding that DPAP B is active in the ER (Roberts *et al.*, 1989). The location of the hydrophilic domain suggests that DPAP B has a cytoplasmic domain of 29 amino acids. Previous reports indicated that DPAP B has a molecular mass of 40 kDa (Garcia-Alvarez *et al.*, 1985) rather than the protein product described subsequently and shown to have DPAP B activity. This discepancy can be explained by assuming that the 40 kDa species results from proteolysis duing purification, or there is another dipeptidyl aminopeptidase activity present in *S. cervisiae*. The sorting signal of DPAP B is unknown, in fact it has been suggested that sorting of vacuolar membrane proteins occurs by default without any sorting information (Raymond *et al.*, 1992).

Other Proteinaceous Constituents. Other proteins having reportedly been found associated with the vacuole are acetylesterase, acid phosphatase, exo-β-1,3-gluconase, invertase, uricase, polyphosphate kinase and catalase A (Cooper, 1982; Schwencke, 1991). The majority of enzymes have been characterized by their enzymatic activity, and in some cases (e.g. catalase A) their existence is doubtful (Wiemken *et al.*, 1979). It is interesting to note the periplasmic proteins invertase and acid phosphatase (Schwencke *et al.*, 1991), exo-β-1,3-glucanase (Cortat *et al.*, 1972), p-nitrophenyl-acetate esterase (Schwencke, 1991) and chitinase (Schwencke, 1991) present in the vacuole. Their presence in the vacuole might be due to accidential packaging into vacuole destined transport vesicles and/or by endocytosis of the secreted enzymes or considered doubtful.

4.1.12. Mutants Defective for Vacuolar Biogenesis

The diversity of vacuolar functions suggest the existence of a sophisticated mechanism of control at many stages, and a number of mutational approaches have

been taken to examine aspects of vacuole biogenesis. The number of approaches are almost as numerous as the vacuolar protein constituents. A surprising finding was that most genes involved in one aspect of vacuole biogenesis were also defective in other related or unrelated aspects of vacuole biogenesis which led to an extensive overlap of the genetic complementation groups identified (Raymond *et al.*, 1992). This phenomenon is due to overlap of different aspects of vacuolar function, and thus prevents the unambiguous identification of whether a defect is primary or secondary. The following sections first describe the different approaches taken to isolate biogenesis mutants, and an effort is made in the last section to compile all available data on proteins that have been identified as participants in vacuolar biogenesis.

4.1.13. Isolation and Characterization of Vacuolar Protein Sorting Mutants

Since the majority of vacuolar proteins known so far enter the endoplasmic reticulum and transgress the secretory pathway in transit to the vacuole, this process of biogenesis has received most attention (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). Based on the findings that secretion is the default pathway for soluble proteins that have entered the ER if no other retention or targeting information is present, and that proteins with vacuolar sorting information are subject to a saturable, active sorting process, strategies have been devised to isolate mutants which mislocalize vacuolar proteins to the cell surface (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989).

To isolate mutants defective in the selective sorting of the vacuolar CPY two approaches have been designed to allow the selection for mutants mislocalizing CPY to the cell surface. One protocol selected for the secretion of a CPY-invertase fusion in Δ*suc2* strains of *S. cerevisiae* (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). This selection procedure took advantage of the fact that a gene fusion consisting of a 433-residue amino-terminal domain of CPY and a 511-residue carboxy-terminal domain of the secretory invertase (pCYI433) is quantitatively located to the vacuole. If the sorting process is affected by a mutation in a gene involved in sorting or vesicle budding in the

sorting compartment, or in the N-terminus of the pro-peptide of the gene fusion, the fusion peptide will be secreted. Δ*suc2* strains of *S. cerevisiae* harbouring the pCYI433 plasmid are unable to grow on media containing sucrose as sole carbon source because the only form of invertase is in the form of the fusion peptide located in the vacuole. Yeast is generally unable to transport sucrose into the vacuole efficiently. If the vacuolar sorting process is affected by a mutation, the fusion will be localized to the cell surface, and such mutants will be able to grow on sucrose medium. This procedure has been used to identify a total of 33 complementation groups termed *vpt* for *v*acuolar *p*rotein *t*argeting mutants (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). The second approach taken was based on the fact that the extracellular fraction of oversecreted p2CPY is proteolytically activated by an unknown periplasmic protease not identical to Barlp (Stevens *et al.*, 1986). Since vacuolar CPY in a *pep4*-3 mutant is not matured to an active form, but secreted p2CPY is, this fact has been used to design a mutant isolation scheme (Rothman and Stevens, 1986; Rothman *et al.*, 1989). Enzymatically active CPY is able to catalyze the peptide bond cleavage of the N-blocked dipeptide N-CBZ-L-Phe-L-Leu (Kuhn *et al.*, 1974; Wolf and Weiser, 1977). Leucine auxotrophs that have normal levels of active vacuolar CPY are able to maintain growth on medium containing N-CBZ-L-Phe-L-Leu as sole leucine source (Rothman and Stevens, 1986). Leucine auxotrophs carrying a *pep4*-3 mutation are unable to grow on medium containing the dipeptide as sole leucine source because intracellular CPY cannot be proteolytically activated (Jones *et al.*, 1982). Overproduction induced secretion of p2CPY led to an activation of the extracellular CPY fraction, and the *leu2 pep4*-3 auxotrophs were able to grow (Rothman and Stevens, 1986). These findings were used to isolate mutants that secrete significant amounts of p2CPY as a result of a vacuolar mislocalization defect. A total of 29 *vpl* recessive complementation groups (for *v*acuolar *p*rotein *l*ocalization) were identified by this procedure (Rothman and Stevens, 1986; Raymond *et al.*, 1992; Rothman *et al.*, 1989; C.K. Raymond, I. Howald and T.H. Stevens, unpublished results). The *VPL* and *VPT* complementation groups have been given the common nomenclature *VPS* (for *v*acuolar *p*rotein *s*orting) because complementation analysis between both sets of mutants established that a large number

are identical (Robinson *et al.*, 1988; Rothman *et al.*, 1989). Other mutants in different aspects of vacuolar function also show an extensive overlap with *vps* mutants, suggesting that many vacuolar functions overlap or are interdependent (see below). These *vps* mutants belong to 46 recessive complementation groups, indicating that a large number of gene products are directly or indirectly responsible for the correct and efficient sorting of vacuolar enzymes. A repeated complementation analysis of the *vps* phenotype demonstrated that *VPS7* is identical to *VPS34*, and *VPS40* to *VPS15* (Raymond *et al.*, 1992).

A comprehensive pheonotypic analysis of the *vps* mutants established that all mutants exhibited defects in the sorting and processing of several vacuolar hydrolases, namely CPY, PrA, PrB and α-mannosidase (Robinson *et al.*, 1988). 19 of the 33 original *vpt* alleles had extreme defects in that they secreted more than 50% of newly synthesized p2CPY and were unable to intracellularly mature the remaining p2CPY to its mature form. The fact that all so far examined *vps* mutants secrete the p2CPY precursor is indicative for a sorting defect at a stage immediately following the Golgi-dependent processing of CPY and before the CPY precursor reaches the vacuole. The fact that the p2CPY precursor accumulates in *sec1*-1 *vps* double mutants at 37°C and is not secreted suggests that the sorting occurs at a stage in the Golgi apparatus where secretory vesicles are generated, and that the p2CPY is secreted via secretory vesicles or in other vesicles in a parallel pathway which also require the *SEC1* gene product for secretion of its contents (Rothman and Stevens, 1986). Vacuolar membrane sorting was generally unaffected in most mutants except in alleles of four complementation groups where up to 50% α-mannosidase was mislocalized to the cell surface (Robinson *et al.*, 1988). This finding is interesting because subsequent studies demonstrated that α-mannosidase does enter the vacuole directly from the cytoplasm (Yoshihisa and Anraku, 1990). Several complementation groups have *ts* alleles which co-segregated with the corresponding *vps* alleles, but the *vps* phenotype itself was not temperature sensitive (Robinson *et al.*, 1988). Most mutants did secrete multiple soluble proteins but with four exceptions no membrane associated proteins, suggesting the existence of a common vacuolar protein sorting apparatus, at least for soluble vacuolar proteins

(Rothman *et al.*, 1989). However, the extent of secretion of PrA and PrB precursors is not as obvious as that of CPY (Robinson *et al.*, 1988), and it appears that instead the precursors accumulate in a compartment not identical to the vacuole (Klionsky *et al.*, 1992). Recent findings suggest that there are several independent sorting mechanisms even for soluble vacuolar hydrolases (Paravicini *et al.*, 1992) because a *vps35* mutant is only defective in the localization of CPY but not PrA or ALP. The Vps35 gene product is apparently not a sorting receptor and thus it is likely that different vacuolar proteins are sorted by a different mechanisms. It is believed that in some *vps* mutants the majority of CPY is secreted while PrA and PrB accumulate in an unknown compartment where they remain inactive. This compartment and the mechanism for selective retention has not yet been elucidated (Klionsky *et al.*, 1990). Experimental data suggest that vacuolar membrane proteins are faithfully localized to the vacuole except in four *vps* alleles of mutants that do not have a morphologically distinct vacuole (Klionsky and Emr, 1989). This suggests that different sorting mechanisms for soluble and membrane associated vacuolar proteins exist but that both types at least initially migrate through the same compartment.

Light and electron microscopic techniques have been used to assess the vacuolar morphology in *vps* mutants (Banta *et al.*, 1988). Three distinct vacuolar morphology classes have been observed: Class A *vps* mutants in 26 complementation groups contained one to three vacuoles morphologically indistinguisable from vacuoles in the isogenic wild type strain, suggesting that only a fraction of vacuolar proteins, that are not required for maintenance of a vacuolar structure, are mislocalized to the cell surface. Class B vacuolar mutants in three complementation groups have fragmented vacuoles, typically 20 to 40 individual small vacuole-like compartments in one cell. Lastly, class C vacuolar mutants in four complementation groups lack any organelle resembling a vacuole. Instead they accumulate structures with similarity to previously indentified Berkeley-bodies (Novick *et al.*, 1981) resembling an aberrant Golgi oganelle. The nature of the vacuolar defects is not understood, and the interpretation of the results is merely the subject of speculation. It has been suggested before that class C mutants have a *vps* phenotype due to a lack of the target structure vacuole, or that the

lack of the vacuole is a consequence of an extreme sorting defect (Klionsky *et al.*, 1990). The vacuolar morphology of all *vps* complementation groups have recently been reassessed by using indirect immunofluorescence as a means to determine vacuolar morphology and the correct assembly of the vacuolar ATPase (Raymond *et al.*, 1992). The authors assigned the *vps* mutant collection to six vacuolar morphology classes: Class A mutants exhibit wild type morphology in that anti-Pho8p and anti-60 kDa H^+-ATPase subunit localize to the vacuolar membrane. By indirect immunofluorescence the vacuole(s) are clustered in one region of the cell, segregtion patterns are observed that extend from mother cells into daughter cells. In addition, vital staining with CDCFDA are similar to the wild type, and vacuoles can positively been stained with quinacrine, suggesting a normal acidification of the vacuole. *vps2*, *vps8*, *vps12*, *vps13*, *vps30*, *vps32* and *vps44* belong into this group, as do *pep1*, *pep9* and *pep16*. Class B mutants have multiple, dispersed vacuolar compartments as judged by inspection with Normarski contrast microscopy, which are often not recognized by immunofluorescence staining using either antibody because of their relative abundance. Vacuolar segregation structures were not observed but the presence of vacuolar material in newly emerging buds argues for segregation to occur. Multiple small vacuoles were observed by CDCFDA, and quinacrine is accumulated indicative for correct acidification. *vps5*, *vps10*, *vps17*, *vps41*, *vps42*, *vps43*, *vps46*, *pep2* and *pep13* belong to this group. Some genetic interaction has been observed between *vps5*, *vps39* and *vps42*, which suggests that in these three class B mutants the defect of vacuolar biogenesis is identical or that the three mutant gene products interact (I. Howald-Stevenson and T.H. Stevens, unpublished results). Class C mutants have a diffuse anti-Pho8p and anti-60 kDa fluorescence pattern characteristic of the absence of a vacuolar membrane, and CDCFDA staining is evenly distributed throughout the cell. *vps11*, *vps16*, *vps18* and *vps33* belong to this group. Class D morphology exhibiting mutants have a normal vacuole according to its anti-Pho8p fluorescence pattern, but show a vacuolar segregation defect in that segregation structures are hardly observed, and many buds and daughter cells have the appearance of class C vacuolar mutants. The absence of a spherical anti-60 kDa staining pattern, together with the finding that quinacrine in these

mutats does not cause any fluorescence is consistent with a defect in assembly and function of the vacuolar ATPase. *vps3*, *vps6*, *vps9*, *vps15*, *vps19*, *vps21*, *vps26*, *vps29*, *vps34*, *vps35*, *vps38*, *vps45* and *pep11* belong into this group. Class E mutants are the largest group of vacuolar mutants and appear to have normal vacuoles as determined by anti-Pho8p fluorescence. The anti-60 kDa labelling localizes the vacuolar ATPase subunit to a smaller compartment in proximity to the vacuole, which is supported by the fact that most quinacrine labelling is localized next to the vacuole and only moderately to the vacuole. These data are consistent with the aberrant localization of functional vacuolar membrane ATPase to a non-vacuolar, membrane-enclosed compartment. *vps4*, *vps14*, *vps20*, *vps22*, *vps23*, *vps24*, *vps25*, *vps27*, *vps28*, *vps31*, *vps36*, *vps37* and *vps39* belong into this class. *vps1* has been placed into its own vacuolar morphology class, because it has one large central vacuole surrounded by a series of much smaller compartments as judged by its anti-Pho8p and anti-60 kDa fluorescence patterns.

Apart from the severe morphological defects, and a vacuolar acidification defect in several *vps* alleles, members of seven complementation groups also were osmosensitive in that they are unable to grow on YPD medium supplemented with 1.5 M NaCl, 2.5 M glycerol or 1 M KCl (Banta *et al.*, 1988). All alleles of mutants belonging to class C complementation groups, alleles of one class B complementation group, and alleles of two class A complementation groups were osmosensitive, which has been interpreted that the vacuole plays an essential role in osmoregulation. Other pleiotropic effects, such as poor growth on non-fermentable carbon sources, poor growth in minimal medium containing proline as sole nitrogen source, poor sporulation of homozygous diploids, and low frequencies of DNA transformation have been observed. This indicates that the vacuole is involved in a number of interdependent functions, which are no longer maintained if a mutation in vacuole biogenesis prevents the formation of a vacuole. Alternatively, a mutation in a gene required for one vacuolar function might disturb the equilibrium of vacuolar function and as a consequence lead to a pleiotropic defect resulting in the collapse of the vacuole as an organelle. All class C mutants isolated so far are viable at 30°C but are inhibited at a growth temperature of 37°C. The other associated defects of the mutants, including their *vps* phenotype, are

not conditional. This suggests that a function of the vacuole or of the mutated protein is required for maintaining cell growth at elevated temperature (Banta *et al.*, 1988).

4.1.14. Mutants Deficient in the Maturation of Vacuolar Proteinases

Mutants isolated on the basis of reduced levels of vacuolar hydrolases (Jones, 1977) have subsequently been shown to be mostly identical to alleles of *vps* mutants (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). The fact that N-acetyl-DL-phenylalanine β-naphthyl ester is a known substrate for CPY (Wolf and Fink, 1975) has led to the development of a chromographic assay to screen mutants for reduced CPY activity. These *pep* mutants (*pep*tidase-deficient) fall into 17 complementation groups (Jones, 1977; Jones *et al.*, 1981). Althogh the mutagenic analysis was intended to assess the role of PrA, PrB and CPY in protein degradation and turnover, subsequent analysis showed that with the exception of *PEP4* which defines the genetic locus for PrA (Ammerer *et al.*, 1986; Rothman *et al.*, 1986; Woolford *et al.*, 1986), all other *pep* mutants have a *vps* phenotype (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). This suggests that the *pep* phenotype originally described is a secondary consequence resulting from mislocalization of several vacuolar proteinases, which do not activate intracellular CPY. 10 complementation groups are identical to subsequently identified *vps* complementation groups (Raymond *et al.*, 1982). So far unaccounted for are *pep1*, *pep2*, *pep9*, *pep11*, *pep13* and *pep16*, all of which have a *vps* mutant phenotype and complement the *vps* mutant collection (Raymond *et al.*, 1992). Apart from a *vps* phenotype some *pep*-mutants are defective in other aspects of vacuolar biogenesis (see above).

4.1.15. Endocytosis Mutants

Two mutants, *end1* and *end2*, allegedly defective in endocytosis of lucifer yellow have been isolated (Chvatchko *et al.*, 1986). An initial characterization demonstrated that *end1* was defective for the internalization of α-factor, suggesting a defect in receptor mediated endocytosis also. *end1* appeared to accumulate invaginations of the

plasma membrane and did not have any apparent visible vacuole, while *end2* accumulated an internal membrane bound organelle. Complementation analysis (Robinson *et al.*, 1988) and the subsequent finding that the *end1* mutant is able to endocytose α-factor but is unable to degrade it, demonstrated that *end1* is not a endocytosis mutant but identical to *vps11* (Dulic and Riezman, 1989). The lack of α-factor degradation has been attributed to the lack of the vacuole which plays a prominent role in the degradation of α-factor in wild type cells (Riezman, 1985). The *end1* allele of *VPS11* has all other pleiotropic defects associated with the lack of an intact vacuole as described above (Dulic and Riezman, 1989; Dulic and Riezman, 1990). It is defective in gluconeogenesis as well as in aspects of the pheromone response. *end2* is also defective for α-factor degradation, but not endocytosis (Riezman *et al.*, 1986). The defect is not a result of a vacuolar biogenesis defect as in *end1*, but rather a defect of fusion of intermediate vacuolar compartments (endosome) with the vacuole. The morphologically aberrant organelle accumulating in *end2* cells could represent this organelle (Riezman *et al.*, 1986). The subsequent isolation of novel endocytosis mutants has been undertaken but has not been published (Riezman *et al.*, 1986).

4.1.16. Acidification Defective Mutants

A mutant, *vph1*-1, deficient in the acidification of the vacuole was isolated using the fluorescent vacuole specific marker 6-carboxyfluorescein diacetate as an indicator of vacuolar pH (Preston *et al.*, 1989). The vacuolar pH in the mutant is 6.91 as opposed to a pH of 6.17 in wild type cells when determined by pH/fluorescence flow cytometry. The mutant has no effect on vegetative growth.

4.1.17. Calcium Sensitive Mutants

Mutants in 18 complementation groups sensitive to 100 mM Ca^{2+} in the growth medium were isolated and characterized (Ohya *et al.*, 1986). Five complementation groups, *cls7* to *cls11*, have a Pet^- phenotype additionally to their lesions in intracellular

calcium homeostasis (Ohya *et al.*, 1991). Genetic and biochemical studies were able to demonstrate that their phenotype is related to defects in the vacuolar membrane H^+-ATPase, and these mutants are defective in their ATP-dependent Ca^{2+}-uptake. Complementation analysis showed that *CLS7* is identical to *VMA3*, the structural gene for subunit *c* (16 kDa) of the vacuolar ATPase, *CLS8* to *VMA1*, the structural gene for subunit *a* (70 kDa), *CLS9* to *VMA11* (17 kDa subunit), *CLS10* to *VMA12*, and *CLS11* to *VMA13*. Other *cls* mutants are identical with other vacuolar biogenesis mutants. *cls13* is allelic to *vam1*, *vps11*, *pep5* and *end1*. *cls14* is allelic to *slp1*, *vam5*, *vps33* and *pep14*. *cls17* is allelic to mutations in *vam9* and *vps16*, and *cls18* is allelic to *vam8*, *vps18* and *pep3*. Apart from *CLS4*, allelic to *CDC24*, the other *CLS* gene products have not been assigned a function yet, and it is possible that they are also defective in a vacuolar function. The mutational analysis of sensitivity towards Ca^{2+} has proven the vacuolar participation in calcium homeostasis, and also demonstrated that several vacuolar functions are not separable. The fact that vacuolar ATPase mutants have a Pet^--phenotype (Ohya *et al.*, 1991) is interesting because the phenotype indicates that the mutants are unable to carry out gluconeogenesis. Judging from this finding acidification or another vacuolar membrane ATPase function might be required to carry out or regulate gluconeogenesis, possibly through an effect on Pho8p function (see above).

4.1.18. Amino Acid Storage Mutants

Of the 10 amino acids sequestered by the vacuole, lysine is taken up by only one specific transport system which is specific for it and arginine. This and the finding that the cytoplasmic lysine concentration is always maintained below 10 mM by uptake into the vacuole, led to the isolation of mutants which could not sequester lysine into the vacuole (Kitamoto *et al.*, 1988b). One mutant, *slp1*, does not grow well in medium supplemented with 10 mM lysine and has other pleiotropic defects of vacuole function such as sensitivity to Ca^{2+} and histidine, small vacuolar pools for amino acids, a class C vacuolar phenotype and reduced enzymatic activities for PrA, PrB and CPY. Subsequent studies were able to demonstrate that *SLP1* and *VPS33* are identical genetic

loci (Robinson *et al.*, 1988; Wada *et al.*, 1990). The fact that only one *slp* mutant was isolated does not indicate that it is the only mutant with a small lysine pool, because other class C vacuolar mutans share this phenotype (Klionsky *et al.*, 1990).

4.1.19. Vacuolar Morphology Mutants

An initial report (Wada *et al.*, 1988) describes the isolation of mutants with morphologically altered vacuoles in two principal approaches. The first approach relies on treating mutagenized cells with chloroquine, an acidophilic reagent, which is accumulated in the vacuole and causes cell death. Other candidates were selected on the basis that the red fluorophore in *ade1* mutants is accumulated in an intact vacuole, but not in *ade1* strains lacking a vacuole. The mutants fall into 9 complementation groups, *vam1* to *vam9* (*va*cuolar *m*orphology). *vam1*, *vam2*, *vam5*, *vam6*, *vam8* and *vam9* are identical to *vps11*, *vps41*, *vps33*, *vps42*, *vps18* and *vps16*, respectively (Raymond *et al.*, 1992). *vam3*, *vam4* and *vam7* are so far unaccounted for and may represent mutants in three novel vacuolar morphology genes. Their phenotypic analysis has not yet been reported.

4.1.20. Vacuolar Segregation Mutants

Initial studies addressing the inheritance of a vacuolar compartment from mother cell to daughter bud were rather controversial, because they suggested that the vacuole disintegrates before being inherited (Wiemken *et al.*, 1970). Recent studies showed that the vacuole in the mother cells remains intact during budding and that each daughter bud receives some maternal material shortly after bud emergence (Weisman *et al.*, 1987). The vacuolar segregation structure has the shape of a tubule or stream of larger vesicles which derive from the large vacuole in the mother cell and are transported to the bud site (Gomes de Mesquita *et al.*, 1991; T. Vida, personal communication; C.K. Raymond, unpublished results). Different strains of yeast have different segregation features (Gomes de Mesquita *et al.*, 1991). Mutants defective in vacuolar segregation have been isolated by screening *vps*-like mutants for mutants exhibiting temperature

sensitive growth and showing no vacuolar segregation (Weisman *et al.*, 1990). The isolation of segregation mutants was based on the assumption that segregation deficient cells would secrete vacuolar precursors and also be temperature sensitive because the vacuole has been shown to be required for growth at elevated temperature (Banta *et al.*, 1988). Four mutants, *vac1*-1 to *vac4*-1 have beens characterized. *vac1*-1 is identical to *vps19* and *pep7*, *vac3*-1 to *vps36* and *vac4*-1 to *vps15* (I. Howald-Stevenson and T.H. Stevens, unpublished results). Only the *vac2*-1 mutant appears to be unique, and although isolated on the basis of a *vps*-like phenotype, it does not secrete vacuolar proteinases but matured all CPY intracellularly with wild type kinetics, suggesting that vacuolar segregation and missorting are two unrelated processes (Shaw and Wickner, 1991). The vacuolar segregation defect is temperature sensitive, and the vacuole fails to segregate at 37°C. The *VAC2* gene is thought to encode a component of the segregation apparatus (Raymond *et al.*, 1992; Shaw and Wickner, 1991). The *vac1*-1 mutant is not deficient in nuclear division nor mitochondrial segregation, yet it is defective for vacuole segregation at both the permissive and restrictive temperature (Weisman *et al.*, 1990). Daughter buds eventually aquire a vacuole, presumably by *de novo* biogenesis from Golgi derived vesicles. The temperature sensitive phenotype has been attributed to the fact that a vacuolar structure but not the *VAC1* gene product is necessary for growth at elevated temperature. Like most other *vps*-mutants, the *vac1*-1 mutant is deficient for growth on medium containing glycerol as a sole carbon source.

4.1.21. Vacuole Biogenesis Genes

To date a large number of mutantions defective in some aspect of vacuole biogenesis and defining some 60 to 70 complementation groups have been isolated (see above). Most of the mutants are defective in the localization of vacuolar proteins - the *vps* phenotype. This stresses the importance of correct protein sorting in vacuole biogenesis that is defined by 52 gene products. This, however, makes it difficult to look for mutants defctive in vacuolar function other than vacuolar protein sorting. The only exception so far has been *vac2*-1, which is a mutation in vacuole segregation and which

does not exhibit a *vps*-phenotype (Shaw and Wickner, 1991). A defect in one gene in vacuole biogenesis often has a pleiotropic effect (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). This is due to the interdependence of a number of vacuolar functions. A good example is the vacuolar ATPase. A deletion mutant in *VMA3*, the 16 kDa subunit in the vacuolar ATPase, is primarily defective in the failure to assemble other ATPase subunits on the vacuolar membrane, which results in the lack of acidification of the vacuolar network (Anraku *et al.*, 1989; Klionsky *et al.*, 1990; Nelson and Nelson, 1990; Ohya *et al.*, 1991; Raymond *et al.*, 1992; Umemoto *et al.*, 1990). The acidification deficiency has a number of physiological consequences, for example vacuolar Ca^{2+} homeostasis is affected which results in the mutant becoming sensitive to elevated concentrations of Ca^{2+} in the growth medium (Ohya *et al.*, 1991). The same defect results in a perturbation of gluconeogenesis, which is probably regulated in the vacuole (Ohya *et al.*, 1991; Plankert *et al.*, 1991). A lack in vacuole acidification also results in the missorting of vacuolar proteins (Klionsky *et al.*, 1992; Rothman *et al.*, 1990). These considerations demonstrate the difficulty in unambiguously identifying the role certain genes play in vacuole biogenesis, and caution has to be applied when interpeting the following information regarding vacuole biogenesis and the hypothetical involvement of genes in its function.

So far 13 VPS and related genes have been cloned and the sequence of 10 have been determined. With one exception, the genes encode gene products with unknown function and no homolygy to previously isolated gene products (Raymond *et al.*, 1992). *VPS1* has been cloned based on complementation of its temperature sensitive phenotype and sequenced. It is identical *SPO15*, an essential gene for meiotic cell division in *S. cerevisiae* (Rothman *et al.*, 1990; Yeh *et al.*, 1991). It encodes a 704 amino acid hydrophilic protein with a predicted molecular weight of 79 kDa, which has been confirmed by immunoblot analysis. The protein has a GTP binding consensus and preliminary biochemical data suggest that it binds GTP (C.K. Raymond, unpublished data). It belongs to the Mx family of proteins and has significant homology (45% identity) to the microtubule bundling protein dynamin (Yeh *et al.*, 1991). A deletion mutant is viable but unable to grow at an elevated temperature of 37°C. It almost

quantitatively secretes newly synthesized CPY and accumulates aberrant structures as detectable by electron microscopy. Vps1p has been localized to the Golgi and seems to perform an essential function in sorting of vacuolar proteins. Whether or not it participates in a microtubule based motor process remains unclear (Scaife and Margolis, 1990). The *Drosophila Shibire* gene encodes the homologue of dynamin and functions in endocytosis (van der Bliek and Meyerowitz, 1991). So far unknown is whether the sporulation deficient phenotype of a *vps1-spo15* mutant is a consequence of a disturbed nuclear division during meiosis (Yeh *et al.*, 1991) or a consequence of the severe *vps* phenotype (Raymond *et al.*, 1992). *pep4* mutants as well as other *vps* mutants are sporulation deficient and one possible explanation is that the *spo* phenotype is a consequence of the lack of active vacuolar proteinases required for the sporulation response (Raymond *et al.*, 1992; Zubenko *et al.*, 1983). A structural dissection of Vps1p suggests that the N-terminal GTP-binding motif is required for biological function involving GTP-hydrolysis and the C-terminal domain functions in membrane attachment and is also required for biological function (Raymond *et al.*, 1992). Its role in vacuole morphogenesis is not known.

Mutations in *VPS3* are deficient in the sorting of soluble vacuolar proteins and the acidification of the vacuole (Rothman and Stevens, 1986; Rothman *et al.*, 1989). The *VPS3* gene has been cloned from a yeast genomic library selecting for transformants that had a *VPS* phenotype, using N-CBZ-L-Phe-F_3-Leu as growth inhibitor for the *pep4 vps3* strain (Raymond *et al.*, 1990). It has been shown to encode a low abundance, hydrophilic protein of 117 kDa from an open reading frame of 1011 amino acids. It appears to be localized in the cytoplasm althogh its sedimentation behaviour suggests that part of it is associated with a sedimentable subcellular structure not identical to the yeast vacuole. Vps3p migrates as a 140 kDa protein on SDS PAGE suggesting posttranslational modifications. A loss of Vps3p function is accompanied by a missorting of CPY, a vacuolar acidification defect different fom that conferred by mutations in the vacuolar ATPase, and a vacuolar segregation defect. Studies with a *ts* conditional mutant strongly suggest that its primary defect is the sorting of vacuolar proteins. 100-fold overexpression of the protein in yeast resulted in a *vps* defect, and it

induced a class B-like vacuolar phenotype (Raymond *et al.*, 1992). Its mode of action is not known.

VPS11 has been cloned by complementation of the *ts* phenotype of a conditional *end1* mutation (Dulic and Riezman, 1989) and a *pep5* mutation (Woolford *et al.*, 1990). Sequence analysis of the *END1* gene predicted an open reading frame capable of encoding a 1030 amino acid protein with a predicted molecular mass of 118 kDa which is in good agreement with the 120 kDa protein species detected by immunoprecipitation with anti-End1p antibody. The protein product has no overall homology with other proteins but has a short consensus typical for ATP-binding proteins. A deletion mutant did not exhibit any other phenotypes not observed with *end1* alleles, was defective in protein sorting, gluconeogenesis, α-factor degradation and vacuole biogenesis in that it had a class C vacuolar morphology. End1p seems to fractionate with vacuolar membranes in wild type cells, but its biological function is unknown.

VPS15 has been cloned by complementation of its *ts* growth defect (Herman *et al.*, 1991b). The complementing clone rescues all associated defects, including the class C vacuolar morphology of the mutant, and it encodes a protein of 1455 amino acids with a predicted molecular weight of 166 kDa. The protein's 300 N-terminal amino acids have a significant sequence similarity with the catalytic domain of serine/threonine protein kinases, it also has a consensus for attachment of myristic acid at its N-terminus. Subsequent experiments could demonstrate that it is cytoplasmic and myristoylated, verifying the assumption based on protein structure predictions. Vps15p fractionates together with Kex2p, a late Golgi enzyme, suggesting that it is located on the cytoplasmic site of the Golgi apparatus. Site directed mutagenesis of the kinase consensus resulted in an inactive gene product that was unable to function in protein sorting, osmoregulation or growth at high temperature. The same studies presented evidence that Vps15p is autophosphorylated. It also seems to partcipate in the phosphorylation reaction of Vps34p, another protein participating in vacuolar protein sorting (Herman and Emr, 1990; Herman *et al.*, 1991a). Phosphorylation of Vps15p C-terminus appears to be critical for function in sorting at elevated temperature. C-terminal deletions of the protein result in a temperature dependent switch between

accumulation of soluble vacuolar precursors in a saturable intermediate compartment and delivery of the precursors to the vacuole. The point mutation, as well as the deletion mutation does not affect the sorting of vacuolar membrane proteins. The p2CPY accumulating compartment has not been identified, but it might represent an intermediate pre-vacuolar compartment. A morphological analysis of *vps15* revealed that it is vacuolar class D, indicative for a defect in vacuolar ATPase assembly (Raymond *et al.*, 1992). The acidification behaviour of the Vps15p C-terminal deletion mutant has not been examined.

VPS18 has been cloned by complementation of the *ts* growth defect of a *vps18* mutant allele (Robinson *et al.*, 1991) and its CPY deficiency in a *pep3*-12 strain (Preston *et al.*, 1991). The *VPS18* gene encodes a 918 amino acid hydrophilic protein which is associated with the vacuolar membrane in wild type cells (Preston *et al.*, 1991). Unfortunately the subcellular fractionation methods does not exclude the possibility that another compartment of similar density cofractionated with the vacuoles. Vps18p has a cysteine-rich motif near the carboxy terminus which resembels the zinc-finger motif observed with Vps11p and that of a number of other proteins including the post-synaptic protein of rat, the adenovirus E1A protein and Gal4p (Robinson *et al.*, 1991). A deletion of the *VPS18* open reading frame results in the same pleiotropic vacuole related phenotypes observed in the *vps18* mutants. *In vitro* mutagenesis of the cysteine rich motif at the C-terminus led to a temperature-conditional CPY sorting defect but not to a change in vacuolar morphology. The fact that the mutants have a defect in Kex2p dependent α-factor processing, but secrete α-1,3 mannose modified pro-α-factor together with unglycosylated mature α-factor strongly suggests that in the *vps18* mutant the most distal Kex2p containing Golgi compartment is affected by the mutation (Robinson *et al.*, 1991). This compartment has previously been suggested to participate in protein sorting of vacuolar proteins away from secretory proteins. The fact that Vps18p is associated with vacuolar membranes does not exclude the possibility that the protein performs an important pre-vacuolar function. Vps11p and Vps18p have a conserved zinc-finger motif similar to that found in the 43 kDa protein of rat synaptic nerve termini and may be involved in maintaining

the integrity of the *trans* Golgi compartment (Raymond *et al.*, 1992). The fact that both *vps11* and *vps18* strains have no vacuole might be a consequence of a block in Golgi to vacuole membrane flow.

VPS19 has been cloned as *VAC1* by complementation of its temperature sensitive phenotype on medium containing glycerol as sole carbon source (Weisman and Wickner, 1992). The structural gene encodes a 515 amino acid protein with no known homologues but with three separate zink-finger motifs also found in Vps11p and Vps18p. The protein is not essential, although a deletion mutation confers the same vacuolar segregation effect as was observed in the *vac1*-1 allele (Weisman *et al.*, 1990). The subcellular localization as well as the *in vivo* function of Vps19p are not known. Preliminary data suggest that Vps19p plays a direct role in vacuolar segregation since *in vitro* reconstitution of vacuolar segregation depends on functional Vps19p and Vac2p, possibly in concert with the cytoskeleton (T. Vida, open citation).

VPS33 encodes a 691 amino acid protein. It has been cloned by complementation of its temperature sensitive phenotype in a *vps33* mutant (Banta *et al.*, 1990) and of the histidine hypersensitive phenotype in a *slp1*-1 strain (Wada *et al.*, 1990). The gene encodes a 79 kDa hydrophilic protein with no significant homology to previously isolated proteins but with two ATP-binding consensus sequences typically found in ATPases. A disruption of the *VPS33* (*SLP1*) locus was not lethal but resulted in the same aberrant phenotypes as the previously isolated *slp1*-1/*vps33* mutants. Intracellular vacuolar proteins which are not secreted initially accumulate as Golgi modified precursors, although the majority are secreted. One *vps*-mutant had a missense mutation near the consensus for ATP binding which resulted in a temperature conditional phenotype that allowed a vacuole formation at the permissive temperature but lacked vacuoles at the restrictive temperature (Banta *et al.*, 1990). The protein is a relatively rare protein and behaves as a freely soluble cytoplasmic protein in fractionation studies. It has been demonstrated that the ATP-binding of Vps33p is functionally significant, and suggests that the protein is involved in protein traffic from the Golgi to the vacuole. It is phosphorylated, but nothing is known about its function *in vivo*.

VPS34 has been cloned by complementation of a *vps33* temperature sensitive growth defect, using a yeast genomic DNA library, and its sequence determined (Herman and Emr, 1990). It encodes a 875 amino acid protein with a predicted molecular mass of 95 kDa lacking any obvious signal sequence. A disruption of the *VPS34* gene resulted in a temperature sensitive growth phenotype, a vacuole segregation and a severe sorting defect but morphologically normal vacuoles. The protein is a rare protein that *in vivo* associates with a particulate fraction, which can be solubilized with 2 M urea but not with Triton X-100, indicative that the interaction of Vps34p with the pelletable structure occurs by protein-protein interaction. Vps34p might act as a component of a large intracellular structure involved in specific steps of vacuolar protein delivery and vacuolar inheritance. The protein seems to be phosphorylated by Vps15p and *in vitro* experiments suggest that the two proteins associate with each other. The biological function of Vps34p and the role of its interaction with Vps15p is not known (Herman *et al.*, 1991a).

The product of *VPS35* has been analysed in detail (Paravicini *et al.*, 1992). The wild type *VPS35* gene has been isolated by complementation of its vacuolar sorting defects. Its nucleotide sequence has been determined and shown to encode a 937 amino acid protein without any hydrophobic domains or homologies to other proteins. The majority of the protein is associated with a large multiprotein complex. Its association with the complex is saturable in that Vps35p remains cytoplasmic when overproduced. A gene disruption of the *VPS35* locus is not lethal nor confers a temperature sensitive phenotype. The null mutant quantitatively secretes p2CPY but not PrA or PrB and ALP. The differential defect in protein sorting in the mutant is so extreme that all CPY is secreted while the other soluble and insoluble proteins are efficiently located to the vacuole and proteolytically processed. This study provides the first concrete evidence that different pathways for vacuolar protein sorting exist in *S. cerevisiae*. The Vps35p protein product is not a trans-membrane protein but remains associated with the cytoplasmic site of a multiprotein complex probably not associated with a membraneous compartment and thus is unlikely to be a specific sorting receptor for CPY. The precise nature of these findings cannot be explained, but there seem to be

several alternatives to account for this phenomenon. The most likely explanation suggests that different vacuolar proteins might utilize the same transport system to mediate their Golgi to vacuole delivery but utilize different sorting receptors for their selective packaging into a common carrier. Vps35p might function in the specific packaging or modification of the CPY-specific receptor complex (Paravicini *et al.*, 1992).

The nature of other *VPS* genes is as yet unknown, and it seems to be too premature to speculate about the mechanism of vacuole biogenesis from the available information. Some general trends have become obvious: the Golgi apparatus plays a major role in proper vacuole biogenesis, and most if not all mutants not developing a vacuole (class C) seem to have a Golgi associated defect. Several intriguing possibilities seem likely: the class C vacuolar morphology mutants isolated so far do not have a vacuole, and an analysis of a temperature conditional vacuole mutation in *VPS33* showed that the gene product is not required for the maintenance of the vacuole but rather in the formation of a new vacuolar compartment. In addition other class C phenotype conferring mutations are in gene products located in the cytoplasm or between the Golgi aparatus and the vacuole. Thus it is likely that the four class C genes identified so far function in sorting or vesicle budding from the *trans* Golgi compartment. Other *VPS* gene products are either cytoplasmic or associated in multiprotein complexes. This argues that the most important steps of vacuolar protein sorting involves a sophisticated machinery located in the cytoplasm or on the cytoplasmic site of membranes. There is sufficient evidence to suggest that there are differential sorting pathways. One intriguing question remains: in *vps18* the trans-Golgi is impeded in its normal function and yet vacuolar membrane proteins are faithfully retained in the cell although in an inactive form. There are two possibilities: vacuolar membrane proteins are sorted to the vacuole by a default mechanism independent of vesicular transport of soluble proteins to the vacuole, or the proteins are located to the plasma membrane and endocytosed immediately in the mutant and accumulated in endocytotic vesicles.

The nature of the gene products corresponding to the most severe vacuolar biogenesis mutants has revealed that the primary defect of the mutants is in the almost quantitative mislocalization of vacuolar proteins and thus the defects associated with some mutants are of a secondary nature. For example in case of Slp1p which initially was thought to function in regulating lysine uptake or metabolism (Kitamoto *et al.*, 1988a, 1988b) it now seems unlikely that this is the proteins primary defect (Banta *et al.*, 1990). The question arises whether the hypothesized lack of a particular vacuolar function will result in pleiotropic defects, or whether a failure to sort many gene products will also result in a pleiotropic defect. The latter seems to be the more likely hypothesis. This suggests that for future studies in isolating particular vacuolar mutants a double selection procedure has to be developed excluding *vps*-like mutants in the collection, although caution has to be applied because some acidification related mechanisms might have mislocalization as a consequence.

4.1.22. The Isolation and Characterization of Salt-Sensitive Vacuolar Mutants

Several approaches have concentrated on the elucidation of vacuolar structure and function, including the generation of mutants in different aspects of vacuolar function. Some advancement has so far been reported in understanding the pathway soluble vacuolar proteins take. The proposed sorting mechanism for these proteins led to the isolation and characterization of vacuolar protein sorting mutants. Approximately sixty genes participating in the sorting of soluble vacuolar proteins have been identified, and gene products corresponding to eight *VPS* genes have been analysed. It appears that all the genes identified so far act in the cytoplasm or associated with some subcellular compartments. Approaches to identify other vacuolar functions by a reverse genetic approach have been described (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). However, most other genes hypothesized to function in vacuole biogenesis are identical to *VPS* genes, or the mutants have a severe *VPS* phenotype. Considering the fact that vacuolar protein sorting plays a prominent role in supplying the vacuole with its proteinaceous constituents it is not surprizing that mutants that mislocalize vacuolar proteins have

other associated phenotypes. One possible explanation is that other vacuolar mutants which have been isolated on the basis of other phenotypes, as for example their sensitivity to lysine due to the lacking storage function, are identical to *vps* mutants because their primary defect is in sorting which has other consequences. Due to the large number of genes involved in enabling protein sorting to the vacuole it is more likely to identify genes participating in sorting rather than isolating a gene involved in one particular aspect of vacuole biogenesis, because the sorting defect induced similar secondary defects. These considerations led to the experimental approach described in this study to identify novel mutants in one particular aspect of vacuole biogenesis: the proposed role of the vacuole in osmoregulation.

Some of the *vps* mutants isolated do not have a vacuole, or have a large number of small, disrupted vacuoles (Banta *et al.*, 1988). Alleles in four complementation groups (*vps11*, *vps16*, *vps18* and *vps33*) lack any recognizable vacuolar structure, secrete soluble vacuolar proteins and are sensitive to a high osmotic strength in the growth medium (e.g. 1.5 M NaCl) and elevated temperature. Four other osmosensitive *vps* mutants have either disrupted vacuoles (*vps1*), or possess aberrant organelles (*vps15*, *vps34*, *vps40*). All other *vps*-mutants in a *PEP4* background are tolerant to high concentration of osmotically active compounds. These observations indicate that normal vacuolar function might be required for osmoregulation in *S. cerevisiae*, and further that most mutants deficint in sorting maintain osmoregulation, except for those that have severe vacuolar morphology defects.

To study the process of vacuole formation and to collect further evidence for the involvement of the vacuole in osmohomeostasis, an approach was designed to isolate and identify further genes involved in osmoregulation as well as in vacuolar function and biogenesis. All *vps* mutants were selected on the basis of mislocalization of carboxypeptidase Y (Rothman and Stevens, 1986) or a CPY-Invertase fusion system (Bankaitis *et al.*, 1986). Only eight out of 47 complementation groups had alleles with aberrant vacuoles, five of these also had osmosensitive alleles. It was expected that among mutants selected directly for osmosensitivity would be novel mutations with aberrant vacuolar function and structure, but not necessarily with a *vps* phenotype. It

appears to be a general feature of the vacuole to carry out many overlapping functions (Klionsky *et al.*, 1990), and a mutant allele of a gene directly or indirectly connected with one aspect of vacuole sorting or biosynthesis might lead to the expression of other, related mutant phenotypes. Alternatively, osmosensitivity has been reported in cytoskeletal mutants (Novick and Botstein, 1985), plasma membrane ATPase mutants (McCuscer *et al.*, 1987), and perhaps associated with nonsense-suppressors (Singh, 1977), or they might be unable to grow under osmotic stress because they fail to accumulate intracellular glycerol (Blomberg and Adler, 1989). However, most osmosensitive mutants isolated so far in *S. cerevisiae* have not been examined for vacuolar morphology and the secretion of vacuolar proteins, and may have some defective vacuolar function. In addition, initial studies addressing the phenomenon of aquired osmoresistance in yeast suggest that the accumulation of glycerol is one of many mechanisms that control osmohomeostasis in *S. cerevisiae*, and efficient osmoregulation depends on protein synthesis and not solely to synthesis of glycerol (Blomberg and Adler, 1989). Considering the fact that not every osmosensitive mutation will have a vacuolar defect, it was decided to isolate osmosensitive mutants and screen the mutants for vacuole associated defects, such as vacuolar morphology. Mutants that were strictly osmosensitive and also had one or several vacuolar biogenesis defects associated with it were termed salt-sensitive vacuolar (*ssv*) mutants, whose isolation and characterization is described in the following part.

S. cerevisiae was mutagenized and 97 mutants were obtained which were unable to tolerate high osmotic stress. They were tested for complementation against each other and previously isolated vacuolar mutants to determine the number of putative genes associated with this phenotype. Subsequent examination of vacuolar morphology and vacuolar protein sorting established that most osmosensitive mutants defined new complementation groups and were defective in several aspects of vacuole biogenesis, including vacuolar morphology and the sorting of vacuolar proteins.

4.2. Results

4.2.1. Mutagenesis of SEY6210 and SEY6211

The wild type strains SEY6210 and SEY6211 were mutagenized by either E.M.S. induced mutagenesis at a predicted rate of 50% survival (Fink, 1970) or U.V. mutagenesis at 20% survival. Since the efficiency of U.V. mutagenesis depends very much on the type and age of the light source and the conditions under which the mutagenesis is performed, a dose response curve was constructed as described in Materials and Methods (Figure 4.1). From the dose-response curve it was found that an exposure time of 210 seconds yielded 20% survivors, a value estimated to give an optimal ratio between single and double mutations. Mutagenesis was carried out by placing a 10 mL agitated yeast cell suspension in a 9 cm petri-dish underneath a UV source (254 nm) at a distance of 10 cm from the light source and samples were taken at set intervals (see Materials and Methods). The survival rate was calculated by dividing viable cell counts of the mutagenized culture by the original viable cell count before mutagenesis times 100%.

Analysis of each mutagenized culture showed that the average percentage of survivors after mutagenesis was 13.0% for E.M.S., and 13.4% for U.V. mutagenesis (Table 4.2). The variation between different subcultures of the wild type subjected to the same mutagenesis conditions was quite large. Since different numbers of cells were screened for osmosensitive mutants, a direct comparison of the rate of killing and the number of osmosensitive mutants was not possible. A rough estimate of the number of mutants obtained for each 100 isolated colonies after mutagenesis varied drastically and any obvious relationship describing the mutagenesis efficiency related to dose or mutagen was not detectable.

A total of 339 putative salt sensitive mutants from 13 independent mutagenised cultures were isolated by negative selection of replicas on 1.5 M NaCl-containing YPD medium. Mutagenized colonies grown on YPD agar plates at 30^{o}C for 48 hours were

replica-plated onto YPD medium supplemented with 1.5 M NaCl. The selective plates were incubated at 30°C for 7 days, and the original master plates were kept at 4°C. Out of 4002 colonies (1503 EMS; 2499 UV), 339 (154 EMS; 185 UV) colonies did not grow under the selective conditions as compared to the original master plate. All 339 colonies on the master plate, which were unable to grow on the replica were picked and purified on YPD. After 48 hours incubation at 30°C, single colonies were taken and streaked onto YPD and YPD supplemented with 1.5 M NaCl. Among 339 mutant clones, a total of 97 mutants (54 EMS; 43 UV) were truly salt sensitive, i.e. they were unable to grow on YPDA + 1.5 M NaCl after 7 days incubation at 30°C and did not show any colony growth under surface microscopic examination. Overall, 3.25% ($\chi\sigma_n$=2.7) of all clones recovered after mutagenesis had a truely salt-sensitive phenotype, with no significant differences between the two mutagens used. It was observed that the growth of an even larger proportion of the mutants was affected by the salt concentration used. About 7% of all mutagenized colonies did not show growth on YPD + 1.5 M NaCl until 5 days compared to 3 to 4 days of the wild type, and showed some growth after 6 to 7 days, or they were able to grow more slowly than the wild type as judged by colony size. Any mutant that was able to grow to some degree was not chosen for further study. Although this selection might introduce some bias into the mutational analysis of vacuole biogenesis, it is expected to simplify the cloning of the corresponding genes by complementation, since selection for complementation is less troublesome with strains that are totally sensitive to selective conditions, than those that are able to grow to some degree, even under stringent selective conditions. Additionally, all seven osmosensitive *vps* mutants isolated to date were absolutely inhibited by concentrations above 1.2 M NaCl (Banta *et al.*, 1988; M. Latterich, results not shown). All 97 mutants were assigned strain numbers, reflecting the mutagenesis event and the allele number of the original isolate. Derivatives from culture 1 were MLY0101, MLY0103, MLY0104, MLY0106, MLY0107, MLY0108, MLY0109, MLY0111, MLY0113, MLY0115, MLY0116, MLY0117, MLY0120, MLY0121, MLY0124, MLY0125, MLY0126, MLY0127, MLY0134, MLY0139, MLY0144, from culture 2 MLY0202, MLY0209, from culture 3 MLY0304, MLY0307, MLY0309,

MLY0310, from culture 4 MLY0402, MLY0416, MLY0419, MLY0420, from culture 5 MLY0506, MLY0508, MLY0512, MLY0513, MLY0522, MLY0524, MLY0526, MLY0527, from culture 6 MLY0601, from culture 7 MLY0701, MLY0702, MLY0704, MLY0705, MLY0706, MLY0709, MLY0712, MLY0713, MLY0716, MLY0725, MLY0728, MLY0729, MLY0731, MLY0732, culture 8 MLY0803, MLY0805, MLY0806, MLY0808, MLY0810, MLY0811, MLY0812, MLY0813, MLY0815, MLY0816, culture 9 MLY0902, MLY0905, MLY0908, MLY0911, MLY0913, culture 10 MLY1001, MLY1002, MLY1005, MLY1006, culture 11 MLY1105, MLY1124, MLY1129, MLY1138, MLY1141, MLY1143, MLY1153, MLY1156, MLY1161, MLY1162, MLY1164, MLY1172, MLY1176, culture 12 MLY1203, MLY1214, MLY1218, MLY1219, MLY1232, MLY1233, and culture 13 were MLY1309, MLY1311, MLY1315, MLY1330 amd MLY1332. Strains MLY0527, MLY0304 and MLY0309 were not pure clones as judged by different colony appearence. Subclones derived from the mixed cultures were designated MLY0527A, MLY0527B, MLY0304A, MLY0304B, MLY0309A and MLY0309B. All subclones were osmosensitive.

4.2.2. Complementation Analysis

A genetic complementation test was employed to evaluate the number of genes involved in conferring osmoresistance to the wild type yeast cell that were identified before. Generally, if two recessive mutations in a diploid cell complement by restoring the wild type phenotype they affect different genes. A failure to complement indicates that two mutations are in the same gene or, one of the mutation is in a regulatory element of the other gene, or lastly one of the mutations yields an inhibitory gene product. Two major complementation tests were carried out sequentially, the first being intended to obtain an estimate of how many complemementation groups were defined by the mutants, and also to select out mutants with aberrant complementation behaviour, especially those mutants apparently members of several complementation groups. If any aberrant complementation pattern is genuine and caused by a single gene

mutation, it is potentially interesting in that it is possible to derive information about genetic interaction of gene products involved in osmoregulation.

Selection for diploids between mutants of different mating type was carried out via complementing auxotrophic markers on yeast minimal medium only supplemented with common nutritional requirements. Diploid colonies were replica-plated onto YPD + 1.5 M NaCl. Growth was recorded after 7 days incubation at 30°C, where complementing mutations showed growth under these conditions. The complementation test was carried out three times, and overall the complementation data were in good agreement with each other (Table 4.3). The second complementation tests were performed to examine complementation of mutants of the same mating type. All mutant strains which were thought to be genuine single mutations as judged by their complementation pattern were first crossed against the appropriate wild type, then salt sensitive spores of the opposite mating type were identified after screening random spore isolates for the desired phenotypes. After mating type analysis of at least 5 randomly chosen *ade2* salt-sensitive spore isolates members of opposite mating type were then subjected to complementation analysis (Table 4.4). Every salt sensitive allele of both mating types was crossed against all others in a drop-overlay complementation test. Since the method employed could not rely on the isolation of auxotrophy complementing diploids, log phase cultures of the salt sentive alleles were harvested, and each mutant was crossed against all other mutants in microtiter plates which were incubated at 30°C for 48 hours to allow mating to occur. 5 µL aliquots of each mating mixture were plated out onto YPD medium supplemented with 1.5 M NaCl, and growth was recorded after 14 days. Diploids that failed to grow on the medium were said to either belong to the same complementation group or to be unable to form diploids. All mating mixtures that failed to grow were assessed for their ability to form ascospores under limiting nutrient conditions, and if no ascospores were observable under phase contrast microscopy the result was recorded as ND (no diploids).

The complementation analysis allowed the assignment of the salt sensitive mutants to at least 23 different complementation groups (Table 4.5), which were assigned the complementation allele designation *ssv1* to *ssv20* (for salt sensitive

vacuolar mutants, since each group had alleles deficient in at least one vacuolar function; see following sections), *osm3* and *osm4* (*osm*osensitive growth). Members of *osm3* and *osm4* were only osmosensitive but had no other obvious vacuolar defects. The results of the initial complementation test (Table 4.3) were generally unambiguous, but a number of mutants were unclassifiable. Assuming that they were double mutants, they were not characterized further. 23 mutants showed only weak complementation with a large proportion of the other *ssv* mutants (semi-dominant phenotype). They were assigned an allele number *ssv*-100 to *ssv*-122. As these mutants are all phenotypically very similar and completely fail to complement each other, they might constitute different members of one further complementation group, *ssv17*. Two mutants, both of the **a**-mating type, did not form diploids under any conditions. They were thought to be truly sterile and were designated *ssv*-123 and *ssv*-124. All remaining *ssv* mutants exhibited osmosensitive phenotypes which were recessive in heterozygous diploids. An auxotrophy marker analysis of all once back-crossed strains was carried out, and in general only back-crossed strains were used in future analysis to reduce the risk of their phenotype being the result of several mutations. Where the phenotypic marker differed from the original isolate a new strain designation was given (Table 4.6). Genetic tests on the validity of the first 17 complementation groups carried out by others (I. Howald-Stevenson and T.H. Stevens, unpublished results) suggest that with the exception of *ssv1*-7, which is identical to *vps33*, and *ssv9*-1, which does not belong in the *ssv9* complementation group, the assignment into groups was largely correct, although allelism tests yet have to show the number of genes involved in conferring a *ssv* phenotype.

Culture	% Survivors of Mutagenesis		Total Colonies	1.5 M $NaCl^S$ Mutants	
	E.M.S.	U.V.	Screened	1st Screen	2nd Screen
1	26.0%		651	45	21
2	9.1%		99	11	2
3	9.9%		165	16	4
4	8.8%		158	20	4
5	6.5%		104	27	8
6	4.9%		88	2	1
7	26.4%		238	33	14
8		22.0%	100	16	10
9		16.0%	311	13	5
10		20.0%	270	6	4
11		6.2%	501	76	13
12		9.1%	732	38	6
13		7.2%	585	36	5
Average	13.0%	13.4%			

Table 4.2: Mutagenesis outcome of EMS and UV mutagenesis of parental strains SEY6210 and SEY6211. The mutagenized cultures 1, 8, 9 and 10 were derived from SEY6210, cultures 2, 3, 4, 5, 6, 7, 11, 12 and 13 from SEY6211. A total of 339 1.5 M NaCl sensitive mutants were obtained from 13 independent mutagenesis events (see text).

Figure 4.1: Dose-response curve of SEY6210 exposed to UV light as measure of time. A 10 mL suspension of 9.0 x 10^7 cells/mL was irradiated under conditions as described in the text. 100 μL samples were removed at 15 second intervals, and the viable cell number was determined.

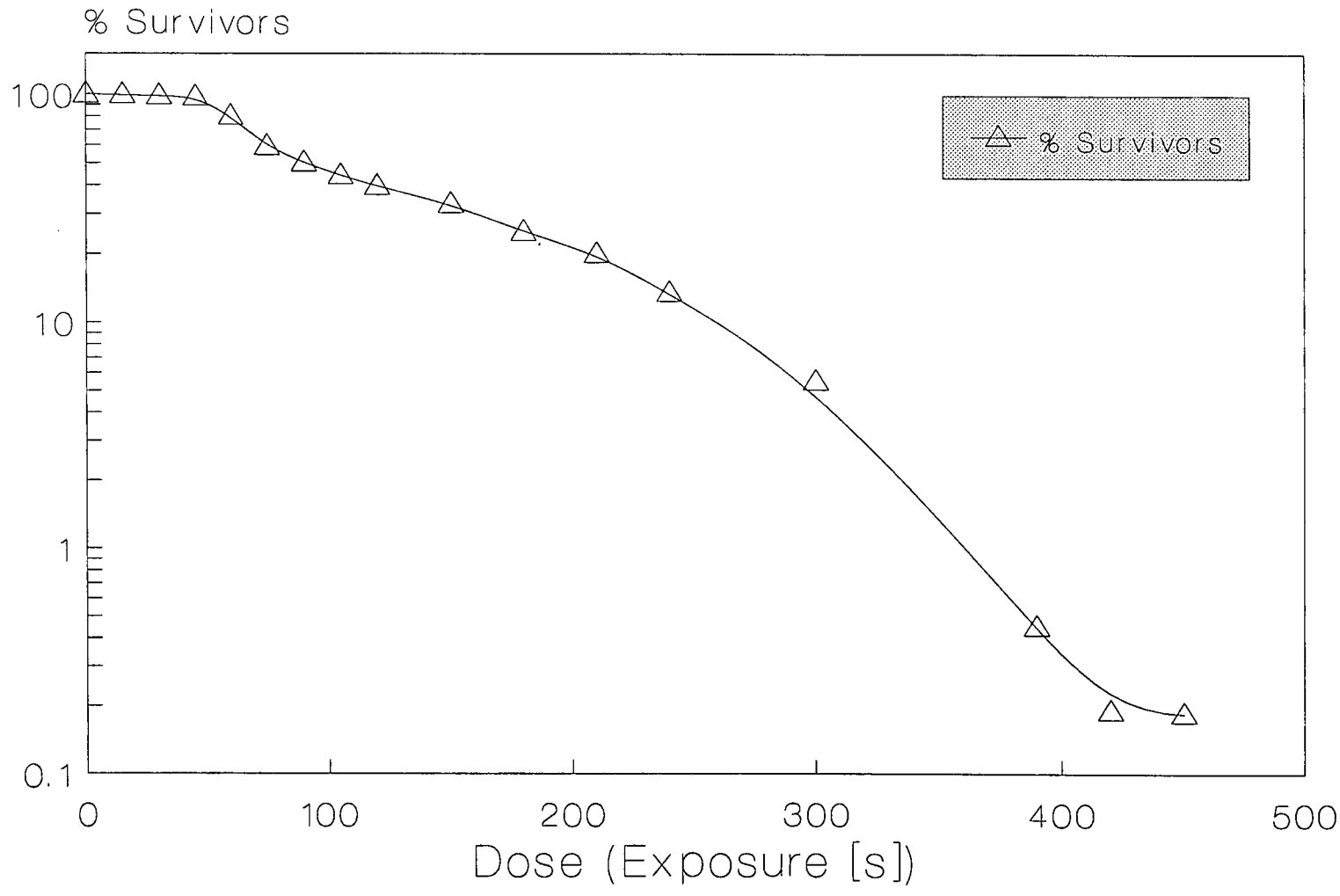

% Survivors
100
10
1
0.1
0
100
200
300
400
500
Dose (Exposure [s])
% Survivors

Table 4.3

a-mutant	SEY6211	MLY0202	MLY0209	MLY0304	MLY0307	MLY0309	MLY0310	MLY0402
α-mutant								
SEY6210	+	ND	+	+	+	+	+	+
MLY0101	+	ND	+	+	+	+	+	+
MLY0103	+	ND	+	ND	+	+	+	+
MLY0104	+	ND	+/-	+/-	-	+	+	+
MLY0106	+	ND	-	-	-	+	+	+
MLY0107	+	ND	+	+	+	+	+	+
MLY0108	+	ND	+	+/-	-	+	+	+
MLY0109	+	ND	+	+/-	+/-	+	+	+
MLY0111	+	ND	+	+	+	+	+	+
MLY0113	+	ND	+	+/-	+/-	+	+	+
MLY0115	+	ND	+	+/-	+/-	+	+	+
MLY0116	+	ND	-	+/-	+	+	-	+
MLY0117	+	ND	+	+	+	ND	+	+
MLY0120	+	ND	+	+/-	+/-	+	+	+
MLY0121	+	ND	ND	+	+	+	+	+
MLY0124	+	ND	ND	+	+	+	+	ND
MLY0125	+	ND	+	+/-	-	+/-	+	+
MLY0126	+	ND	+	+	+	+	+	+
MLY0127	+	ND	+/-	-	+/-	+	+	+
MLY0134	+	ND	-	+	+	+	+	+/-
MLY0139	+	ND	+	+	+	+	+	+
MLY0144	+	ND	+	+/-	-	+	+	+
MLY0145	ND	ND	ND	-	-	ND	+	ND
MLY0803	+	ND	+	+	+	+	+	+
MLY0805	+	ND	+	+/-	+	+	+	+
MLY0806	+	ND	+	+	+	+	+	+
MLY0808	+	ND	+	+	+	+	+	+
MLY0810	+	ND	+	+	+	+	+	+
MLY0811	+	ND	+	+	+	-	-	+/-
MLY0812	+	ND	+	+	+	+	+/-	+
MLY0813	+	ND	+	+	+	+	+	+/-
MLY0815	+	ND	+	+/-	+	+	+	+
MLY0816	+	ND	+	+	+	+	+	+
MLY0902	+	ND	+	+	+	+	+	+
MLY0905	+	ND	+	+	+	+	+	+
MLY0908	+	ND	+	+	+	+	+	+
MLY0911	+	ND	+	+	+	+/-	+	+
MLY0913	+	ND	+	+	+	+	+	+
MLY1001	+	ND	+	+	+	+	-	+
MLY1002	+	ND	+	+	+	+	-	+
MLY1005	+	ND	+	+	+	+/-	-	+
MLY1006	+	ND	+	+	+	+	+	+
vps5	+	ND	ND	+	ND	-	+	ND
vps11	+	ND	ND	+	+	+	+	+
vps16	+	ND	ND	+	ND	ND	ND	+
vps17	+	ND	+	+	ND	+	+	ND
vps18	+	ND	ND	+	ND	+	+	+
vps33	+	ND	ND	+	ND	+	ND	+
B-4164	+	ND	ND	+	+	+	ND	+

Table 4.3 - continued

a-mutant	MLY0416	MLY0419	MLY0420	MLY0508	MLY0512	MLY0513	MLY0522	MLY0524
α-mutant								
SEY6210	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	+	+	+	+	+	+	+	+
MLY0106	+	+	+	+	+	+	+/-	+
MLY0107	+	+	+	+	+	+	+	+/-
MLY0108	+	+	+	+/-	+	+	+/-	+
MLY0109	+	+	+	+	+	+	-	+
MLY0111	+	+	-	ND	+	+	+	+
MLY0113	+	+	+	+	+	+	+/-	+/-
MLY0115	+	+	+	+	+	+	+/-	+
MLY0116	-	+	+	-	+	+	+/-	+
MLY0117	ND	ND	ND	ND	ND	ND	+	ND
MLY0120	+	+	+	+	ND	+/-	+	+
MLY0121	+	+	+	ND	+	+	+	+
MLY0124	+	+	-	ND	+	+	+	+
MLY0125	+	+	+	+	+	+	+	+
MLY0126	+	+	+	+	+	+	+	+
MLY0127	+	+	+	+	+	+	+/-	+
MLY0134	+	+	+	ND	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0144	+	+	+	+/-	+	+	+/-	+
MLY0145	+	-	-	ND	+	+	-	+
MLY0803	+	+/-	+	-	+	+	+	+
MLY0805	+	+/-	+	-	+	+	+	+
MLY0806	+	+/-	+	-	+	+	+	+
MLY0808	+	+	+	-	+	+	+	+
MLY0810	+	+/-	+	-	+	+	+	+
MLY0811	-	+/-	-	-	+	+	+	-
MLY0812	+/-	+/-	+	-	+	+	+	+/-
MLY0813	+	+/-	+	-	+	+	+	+/-
MLY0815	+/-	+/-	+	-	+	+	+	+/-
MLY0816	+	+/-	+	-	+	+	+	+
MLY0902	+	+/-	+	-	+	+	+	+/-
MLY0905	+	+/-	+	-	+	+	+	+
MLY0908	+	+/-	+	-	+	+	+	+
MLY0911	+	+/-	+	-	+	+	+	+
MLY0913	+	+/-	+	-	+	+	+	+
MLY1001	+	+/-	+	-	+	+	+	+
MLY1002	+/-	+/-	+/-	-	+	+	+	-
MLY1005	+	+/-	+/-	-	+	+	+	+
MLY1006	+	+	+	+	+	+	+	+
vps5	+	+	+	+	+	+	+	+/-
vps11	+	+	+	+	+	+	+	+
vps16	+	ND	+	ND	+	+	+	+
vps17	+	ND	ND	+	+	+	+	+
vps18	+	ND	+	+	+	+	+	+
vps33	+	ND	+	+	+	+	+	+/-
B-4164	+	ND	+	+	+	+	+	+

Table 4.3 - continued

a-mutant	MLY0526	MLY0527A	MLY0527B	MLY0601	MLY0701	MLY0702	MLY0704	MLY0705
α-mutant								
SEY6210	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	+	+	+	+/-	+	+	+	+
MLY0106	+	ND	+	+/-	+	+	+	+
MLY0107	+	+	+	+	+	+	+	+
MLY0108	+	+	+	+/-	+	+	+	+
MLY0109	+	ND	+	+/-	+	+	+	+
MLY0111	+	ND	ND	+	+	+	+	+
MLY0113	+	+	+/-	+/-	+	+	+	+
MLY0115	+	+	+	+/-	+	+	+	+
MLY0116	+	+/-	+	+/-	+	+	+	+
MLY0117	ND	ND	+	+	+	-	ND	ND
MLY0120	+	ND	ND	+	+	+	+	+
MLY0121	+	ND	ND	+	+	+	-	+
MLY0124	+	ND	ND	+	+	+	ND	+
MLY0125	+	ND	+/-	+/-	+	+	+	+
MLY0126	+	ND	+	+	+	+	+	+
MLY0127	+	+	+/-	+/-	+	+	+	+
MLY0134	+	ND	+	+	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0144	+	+	+	+/-	+	+	+	+
MLY0145	+	ND	ND	+/-	+	+	+	+
MLY0803	+	+	+	-	+	+	+	+
MLY0805	+	+	+	-	+	+	+	+
MLY0806	+	+	+	-	+	+	+	+
MLY0808	+	+	+	-	+	+	+	+
MLY0810	+	+	+	-	+	+	+	+
MLY0811	+	+	+	-	+	-	+/-	+
MLY0812	+	+	+	-	+	+	+	+
MLY0813	+	+/-	+	+/-	+	+	+	+
MLY0815	+	+	+	-	+	+	+	+
MLY0816	+	+	+	-	+	+	+	+
MLY0902	+	+	+	-	+	+	+	+
MLY0905	+	+	+	-	+	+	+	+
MLY0908	+	+	+	-	+	+	+	+
MLY0911	+	+	+	-	+	+	+	+
MLY0913	+	+	+	-	+	+	+	+
MLY1001	+	+	+	-	+	+	+	+
MLY1002	+	+	+	-	+	+	+/-	-
MLY1005	+	+	+	-	+	+	+	+
MLY1006	+	+	+	+	+	+	+	+
vps5	+	+	+	ND	+	+	+	+
vps11	+	ND	+	+	+	+	ND	+
vps16	ND	ND	-	ND	+	ND	+	+
vps17	+	+	ND	ND	+	+	+	+
vps18	+	ND	+	ND	+	+	+	+
vps33	+	ND	ND	ND	+	ND	+	+
B-4164	+	ND	ND	ND	+	+	+	+

Table 4.3 - continued

a-mutant	MLY0706	MLY0709	MLY0712	MLY0713	MLY0716	MLY0725	MLY0728	MLY0729
α-mutant								
SEY6210	+	+	ND	+	+	+	+	+
MLY0101	+	+	ND	+	+	+	+	+
MLY0103	+	+	ND	+	+	+	+	+
MLY0104	+	+	ND	+/-	+/-	+	-	+
MLY0106	+	+	ND	+/-	+/-	+	-	+
MLY0107	+	+	ND	+	+	+	+	+
MLY0108	+	+	ND	+/-	-	+	-	+
MLY0109	+	+	ND	+/-	+/-	+	-	+/-
MLY0111	+	+	ND	+	+	+	+	+
MLY0113	-	+	ND	+/-	+/-	+	-	+/-
MLY0115	+	+	ND	+/-	+/-	+	-	+
MLY0116	+/-	+	ND	+/-	+/-	+/-	-	+/-
MLY0117	+	ND	ND	+	+	+	+	+
MLY0120	+	+	ND	+	+	+	+	+
MLY0121	+	+	ND	+	ND	+	+	+
MLY0124	ND	+	ND	+	ND	+	+	+
MLY0125	-	+	ND	+/-	+/-	+	-	+/-
MLY0126	+	+	ND	-	+	+	+	+
MLY0127	-	+	ND	+/-	+/-	+	-	+/-
MLY0134	+	+	ND	+	+/-	+	+	+
MLY0139	+	+	ND	+	+	+	+	+
MLY0144	+	+	ND	+/-	+/-	+	-	+/-
MLY0145	+	+	ND	+	+	+	-	+
MLY0803	+	+	ND	-	-	+	-	+
MLY0805	+	+	ND	-	-	+	-	+
MLY0806	+	+	ND	-	-	+	-	+
MLY0808	+	+	ND	-	-	+	-	+
MLY0810	+	+	ND	-	-	+	-	+
MLY0811	+/-	+/-	ND	-	-	-	-	-
MLY0812	+	+/-	ND	-	-	+	-	+
MLY0813	+	+/-	ND	-	-	+	ND	+/-
MLY0815	+	+	ND	-	-	+	-	+
MLY0816	+	+	ND	-	-	+	-	+
MLY0902	+	+	ND	-	-	+	-	+
MLY0905	+	+	ND	-	-	+	-	+
MLY0908	+	+	ND	-	-	+	-	+
MLY0911	+	+	ND	-	-	+	-	+
MLY0913	+	+	ND	-	-	+	-	+
MLY1001	+	+	ND	-	-	+	-	+
MLY1002	+/-	+/-	ND	-	-	-	-	+/-
MLY1005	+	+	ND	-	-	+	-	+
MLY1006	+	+	ND	+	+	+	+	+
vps5	+	+	ND	+	+	+	+	+
vps11	+	+	ND	+	+	+	+	+
vps16	+	+	ND	+	+	+	+	+
vps17	+	+	ND	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	+	ND	+	+	+	+	+
B-4164	+	+	ND	+	ND	+	+	+

Table 4.3 - continued

a-mutant	MLY0731	MLY0732	MLY1105	MLY1124	MLY1129	MLY1138	MLY1141	MLY1143
α-mutant								
SEY6210	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	ND	+	+	+	+	+	+
MLY0104	+	+	+	+/-	+/-	+/-	+/-	-
MLY0106	+	+	+	+/-	+/-	+/-	+/-	-
MLY0107	+	+	+	+	+	+	+	+
MLY0108	+	+	+	+/-	+/-	+/-	+/-	+
MLY0109	+	+/-	+	+/-	+/-	+/-	+/-	-
MLY0111	+	ND	+	+	+	+	+	+
MLY0113	+/-	+	+	+	+/-	+/-	+/-	-
MLY0115	+	+	+	+/-	+/-	+/-	+/-	-
MLY0116	+/-	+	+/-	+/-	+/-	+/-	+/-	-
MLY0117	+	ND	+	ND	+	+	+	+
MLY0120	+	+	+	+	+	+	+	+/-
MLY0121	+	ND	+	+	+	+	+	+
MLY0124	ND	ND	ND	+	+	+	+	+
MLY0125	+/-	ND	+/-	+/-	+/-	+/-	+/-	+/-
MLY0126	+	+	+	+	+/-	+/-	+	+
MLY0127	+	+	+	+/-	+/-	+/-	+/-	-
MLY0134	+	ND	+	+	+	+	+	+
MLY0139	+	ND	+	+	+	+	+	+
MLY0144	+/-	+	+	+/-	+/-	+/-	+/-	-
MLY0145	+	ND	+	+/-	+/-	+/-	+/-	-
MLY0803	+	+	+	+/-	+/-	+/-	+/-	+
MLY0805	+	+	+	+/-	+/-	+/-	+/-	+
MLY0806	+	+	+	+/-	+/-	+/-	+/-	+
MLY0808	+	+	+	+/-	+/-	+/-	+/-	+
MLY0810	+	+	+	+/-	+/-	+/-	+/-	+
MLY0811	+/-	+	+/-	+/-	+/-	+/-	+/-	+
MLY0812	+	+	+	+/-	+/-	+/-	+/-	+
MLY0813	+	ND	ND	+/-	+/-	+/-	+/-	+
MLY0815	+	+	+	+/-	+/-	+/-	+/-	+
MLY0816	+	+	+	+/-	+/-	+/-	+/-	+
MLY0902	+	+	+	+/-	+/-	+/-	+/-	+
MLY0905	+	+	+	+/-	+/-	+/-	+/-	+
MLY0908	+	+	+	+/-	+/-	+/-	+/-	+
MLY0911	+	+	+	+/-	+/-	+/-	+/-	+
MLY0913	+	+	+	+/-	+/-	+/-	+/-	+
MLY1001	+	+	+	+/-	+/-	+/-	+/-	+
MLY1002	-	+	-	+/-	+/-	+/-	+/-	+
MLY1005	+	+	+	+/-	+/-	+/-	+/-	+
MLY1006	+	+	+	+	+	+	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	ND	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	+	+	+	+	+	+	+
B-4164	+	ND	+	+	+	+	+	+

Table 4.3 - continued

a-mutant	MLY1153	MLY1156	MLY1161	MLY1162	MLY1164	MLY1176	MLY1203	MLY1214
α-mutant								
SEY6210	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	+/-	+	+	+/-	+/-	+/-	+/-	+
MLY0106	+/-	+	+	+/-	+/-	+/-	+/-	+
MLY0107	+	+	+/-	+	+	+	+	+
MLY0108	+	+	+	+/-	+/-	+/-	+/-	+
MLY0109	+/-	+/-	+	+/-	+/-	+/-	+/-	+
MLY0111	+	ND	+	+	+	+	+	+
MLY0113	+/-	+/-	+	+/-	+/-	+/-	+/-	+
MLY0115	-	+	+	+/-	+/-	+/-	+/-	+
MLY0116	-	-	+/-	+/-	+/-	+/-	+/-	+
MLY0117	+	ND	ND	+	ND	+	+	ND
MLY0120	+	+	+	+	+	+	+	+
MLY0121	+	+	ND	+	+	+	+	+
MLY0124	+	ND	ND	+	+	+	+	+
MLY0125	+/-	ND	+	+/-	+/-	+/-	+/-	+
MLY0126	+	ND	+	+	+	+	+	+
MLY0127	+/-	+	+	+/-	+/-	+/-	+/-	+
MLY0134	+	ND	ND	+	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0144	+/-	+	+	+/-	+/-	+/-	+/-	+
MLY0145	+/-	ND	+	+/-	+/-	+/-	+/-	+
MLY0803	+	+	+	+/-	+/-	+/-	+/-	+
MLY0805	+	+	+	+/-	+/-	+/-	+/-	+
MLY0806	+	+	+	+/-	+/-	+/-	+/-	+
MLY0808	+	+	+	+/-	+/-	+/-	+/-	+
MLY0810	+	+	+	+/-	+/-	+/-	+/-	+
MLY0811	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-
MLY0812	+	+	+	+/-	+/-	+/-	+/-	+/-
MLY0813	+	+	+	+/-	+/-	+/-	+/-	+
MLY0815	+	+	+	+/-	+/-	+/-	+/-	+
MLY0816	+	+	+	+/-	+/-	+/-	+/-	+
MLY0902	+	+	+	+/-	+/-	+/-	+/-	+
MLY0905	+	+	+	+/-	+/-	+/-	+/-	+
MLY0908	+	+	+	+/-	+/-	+/-	+/-	+
MLY0911	+	+	+	+/-	+/-	+/-	+/-	+
MLY0913	+	+	+	+/-	+/-	+/-	+/-	+
MLY1001	+	+	+	+/-	+/-	+/-	+/-	+
MLY1002	+	+	-	+/-	+/-	+/-	+/-	+/-
MLY1005	+	+	+	+/-	+/-	+/-	+/-	+
MLY1006	+	+	+	+	+	+	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	ND	ND	+	+	+	+	+
vps17	+	+	+	+	+	+/-	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	ND	+	+	+	+	+	+
B-4164	+	+	+	+	+	+	+	+

Table 4.3 - continued

a-mutant	MLY1218	MLY1219	MLY1232	MLY1233	MLY1309	MLY1311	MLY1315	MLY1330
α-mutant								
SEY6210	+	+	+	+	+	ND	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	+/-	+/-	+/-	+	+/-	+	+/-	+
MLY0106	+/-	+/-	+/-	+	+/-	+	+/-	+
MLY0107	+	+	+	+/-	+	+	+	+
MLY0108	+	+/-	+	+	+	+	+	+
MLY0109	+/-	+/-	+/-	+	+/-	+	+/-	-
MLY0111	+	+	+	+	+	ND	+	+
MLY0113	+/-	+/-	+/-	+	+/-	+	+/-	-
MLY0115	+/-	+/-	+/-	+	+/-	+	+/-	+
MLY0116	+/-	+/-	+/-	+/-	+/-	+	+/-	+/-
MLY0117	+	ND	+	+	+	ND	+	+
MLY0120	+/-	+	+	+	+	+	+	+
MLY0121	+	+	+	+	+	ND	+	+
MLY0124	+	+	+	ND	+	ND	+	+
MLY0125	+/-	+/-	+/-	+	+/-	+	+/-	+/-
MLY0126	+	+	+	+	+	+	+	+
MLY0127	+/-	+/-	+/-	+	+/-	+	+/-	+
MLY0134	+	+	+	+	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0144	+/-	+/-	+/-	+	+/-	+	+/-	+/-
MLY0145	+/-	+/-	+/-	+	+/-	ND	+/-	+
MLY0803	+/-	+/-	+	+	+	+	+	+
MLY0805	+/-	+/-	+	+	+	+	+	+
MLY0806	+/-	+/-	+	+	+	+	+	+
MLY0808	+/-	+/-	+	+	+	+	+	+
MLY0810	+/-	+/-	+	+	+	+	+	+
MLY0811	+/-	+/-	+	+	+	-	+	+
MLY0812	+/-	+/-	+	+	+	+	+	+
MLY0813	+/-	+/-	+/-	+	+/-	+	+/-	+
MLY0815	+/-	+/-	+	+	+	+	+	+
MLY0816	+/-	+/-	+	+	+	+	+	+
MLY0902	+/-	+/-	+	+	+	+	+	+
MLY0905	+/-	+/-	+	+	+	+	+	+
MLY0908	+/-	+/-	+	+	+	+	+	+
MLY0911	+/-	+/-	+	+	+	+	+	+
MLY0913	+/-	+/-	+	+	+	+	+	+
MLY1001	+/-	+/-	+	+	+	+	+	+
MLY1002	+/-	+/-	+	+	+	-	+	+
MLY1005	+/-	+/-	+	+	+	+	+	+
MLY1006	+	+	+	+	+	+	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	ND	+	+	+	+	+	+	+
vps33	+	+	+	+	+	ND	+	+
B-4164	+	+	+	+	+	+	+	+

Table 4.3 - continued

a-mutant	MLY1332	*vps1*	*vps8*	D597-6D
α-mutant				
SEY6210	+	+	+	
MLY0101	+	+	+	
MLY0103	+	+	+	
MLY0104	+	+/-	+	
MLY0106	+	-	+	
MLY0107	+	+	+	
MLY0108	+	+/-	+	
MLY0109	+	+/-	+	
MLY0111	+	+	+	
MLY0113	+	+/-	+	
MLY0115	+	+/-	+	
MLY0116	+	-	+	
MLY0117	ND	+	ND	
MLY0120	+	+/-	+	
MLY0121	+	+	ND	
MLY0124	+	+	ND	
MLY0125	+	+/-	+/-	
MLY0126	+	+	+	
MLY0127	+	-	+	
MLY0134	+	+	+	
MLY0139	+	+	+	
MLY0144	+	+/-	+	
MLY0145	+	-	ND	
MLY0803	+	+	+	
MLY0805	+	+	+	
MLY0806	+	+	+	
MLY0808	+	+	+	
MLY0810	+	+	+	
MLY0811	+	+	+	
MLY0812	+	+	+	
MLY0813	+	+	+	
MLY0815	+	+	+	
MLY0816	+	+	+	
MLY0902	+	+/-	+	
MLY0905	+	+	+	
MLY0908	+	+	+	
MLY0911	+	+	+	
MLY0913	+	+	+	
MLY1001	+	+	+	
MLY1002	+	+	+	
MLY1005	+	+	+	
MLY1006	+	+	+	
vps5	+/-	+	ND	+
vps11	+	+	+	+
vps16	+	ND	+	+
vps17	+	+	+	+
vps18	+	+	+	+
vps33	+	+	ND	+
B-4164	+	+	+	-

Table 4.3: Complementation of the osmosensitive growth phenotype of the original mutant isolates and previously isolated salt sensitive strains. Every mutant of **a**-mating type was crossed against every mutant of α-mating type and auxotrophy complementing diploids were isolated. Diploids were replica-plated onto YPD supplemented with 1.5 M NaCl, and growth or non-growth under selective conditions was scored after 7 days incubation at 30°C. Two mutant strains were said to complement if the diploid was able to maintain growth under selective conditions (+). Two mutants were said to not complement if diploids failed to grow at all under the selective conditions (-). Some diploid strains showed an intermediate phenotype in that they grew slowly but not as well as a homozygous wild type diploid strain (+/-). Certain other mutants failed to form diploids in the drop overlay complementation test and their complementation pattern could not be tested (ND = no diploids). The scores recorded in the table are the results of three independent complementation tests, and generally were in good agreement in that they were identical in most cases. Strains that failed to complement the NaCl sensitive phenotype of other strains were placed in one complementation group. A number of strains had an aberrant complementation pattern in that they showed weak complementation against other osmosensitive mutants but were fully complemented by the wild type. Since their complementation patterns were similar they were placed into one complementation group (see text). Mutants that occurred in several complementation groups were thought to be double mutants and were eliminated from the groups in which they occurred.

Table 4.4

α-mutant / **a**-mutant	SEY6210	MLY0101	MLY0103	MLY0104	MLY0106	MLY0108	MLY0111	MLY0117
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	-	+	+	+	+	-	-
MLY0103	+	+	-	+	+	+	+	+
MLY0104	+	+	+	-	-	-	+	+
MLY0106	+	+	+	+/-	-	-	+	+
MLY0108	+	-	+	+/-	-	-	+	ND
MLY0111	+	-	+	-	+	+	-	-
MLY0117	+	-	+	+	+	+	-	-
MLY0120	+	+	+	+	+	+	+	+
MLY0124	+	+	+	+	+	+	+	+
MLY0126	+	+	+	+	+	+	+	+
MLY0134	+	-	+	+	+	ND	ND	ND
MLY0139	+	+	+	+	+	+	+	+
MLY0145	+	ND	+	ND	ND	-	+	ND
MLY0209	+	+	+	+	-	ND	+	+
MLY0304A	+	-	+	+	-	-	+	+
MLY0307	+	+	+	-	-	-	+	+
MLY0309A	+	+	+	+	+	+	ND	ND
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	+	+	+	+	+	+
MLY0402	+	+	+	+	+	+	+	+
MLY0416	+	+	+	+	+	+	+	-
MLY0512	+	+	+	+	+	+	+	ND
MLY0513	+	+	+	+	+	+	+	ND
MLY0522	+	+	+	+	+	+	+	+
MLY0524	+	+	+	+	+	+	+	ND
MLY0526	+	+	+	+	+	+	+	ND
MLY0527A	+	+	+	+	+	+	ND	ND
MLY0701	+	+	+	+	+	+	+	+
MLY0704	+	+	+	+	+	+	+	ND
MLY0705	+	ND	+	+	+	+	ND	ND
MLY0706	+	+	+	+	+	+	+	+
MLY0709	+	+	+	+	+	+	+	+
MLY0716	+	+	+	+	+	-	+	+
MLY0725	+	+	+	+	+	+	+	+
MLY0729	+	+	+	+	+	+	+	+
MLY0731	+	+	+	+	+	+	+	+
MLY0732	+	+	+	+	+	+	+	+
MLY1105	+	+	+	+	+	+	+	+
MLY1143	+	+	+	-	-	ND	+	+
MLY1153	+	+	+	+	+	+	+	+
MLY1156	+	+	+	+	+	+	ND	ND
MLY1161	+	+	+	+	+	+	+	+
MLY1214	+	+	+	+	+	+	+	+
MLY1232	+	+	+	+/-	+/-	+/-	+	+
MLY1233	+	+	+	+	+	+	+	+
MLY1311	+	+	+	+	+	+	ND	ND
vps1	+	+	+	+/-	-	+/-	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	ND	ND
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	+	+	+	+	+	+	+
vps34	+	ND	+	+	+	+	+	ND
D597-6D	+	ND	+	ND	ND	ND	ND	ND

Table 4.4 - continued

α-mutant / **a**-mutant	MLY0120	MLY0124	MLY0126	MLY0134	MLY0139	MLY0145	MLY0209	MLY0304A
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	+/-	+	+	+/-	+	+/-	+/-	+/-
MLY0106	+	+	+	+	+	-	-	-
MLY0108	+	+	+	+	+	+/-	+	+/-
MLY0111	+	+	+	+	+	+	+	+
MLY0117	+	+	+	+	+	+	+	+
MLY0120	-	+	+	+	+	+	+	+/-
MLY0124	+	-	+	+	+	+	ND	+
MLY0126	+	+	-	+	+	+	+	+
MLY0134	+	+	+	-	+	+	-	+
MLY0139	+	+	+	+	-	+	+	+
MLY0145	+	+	+	+	+	+/-	+	-
MLY0209	+	+	+	-	+	+	-	+/-
MLY0304A	+/-	+	+	+	+	-	+	-
MLY0307	+/-	+	+	+	+	-	+	-
MLY0309A	+	+	+	+	+	+	+	+
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	+	+	+	+	+	+
MLY0402	+	+	+	-	+	+	+/-	+
MLY0416	+	+	+	+	+	+	+	+
MLY0512	+	+	+	+	+	+	+	+
MLY0513	+/-	+	+	+	+	+	+	+
MLY0522	+	+	+	+	+	-	+	+/-
MLY0524	+	+	+	+	+	+	+	+
MLY0526	+	+	+	+	+	+	+	+
MLY0527A	+	+	+	+	+	+	+	+
MLY0701	+	+	+	+	+	+	+	+
MLY0704	+	+	+	+	+	+	+	+
MLY0705	+	+	+	+	+	+	+	+
MLY0706	+	+/-	+	+	+	+	+	+
MLY0709	+	+	+	+	+	+	+	+
MLY0716	+	+	+	+	+	+	+	+
MLY0725	+	+	+	+	+	+	+	+
MLY0729	+	+	+	+	+	+	+	+
MLY0731	+	+	+	+	+	+	+	+
MLY0732	+	+	+	+	+	+	+	+
MLY1105	+	+	+	+	+	+	+	+
MLY1143	+/-	+	+	+	+	-	+/-	-
MLY1153	+	+	+	+	+	-	+	-
MLY1156	+	+	+	+	+	+	+	+
MLY1161	+	+	+	+	+	+	+	+
MLY1214	+	+	+	+	+	+	+	+
MLY1232	+	+	+	+	+	-	+	-
MLY1233	+	+	+	+	+	+	+	+
MLY1311	+	+	+	+	+	+	+	+
vps1	+	+	+	+	+	+/-	+	-
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	+	+	+	+	+	+	+
vps34	+	+	+	+	+	+	+	+
D597-6D	ND	ND	ND	+	+	ND	ND	ND

Table 4.4 - continued

α-mutant / **a**-mutant	MLY0307	MLY0309A	MLY0309B	MLY0310	MLY0402	MLY0416	MLY0512	MLY0513
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	-	+	+	+	+	+	+	+
MLY0106	-	+	+	+	+	+	+	+
MLY0108	-	+	+	+	+	+	+	+
MLY0111	+	+	+	+	+	+	+	+
MLY0117	+	+	ND	+	+	+	+	+
MLY0120	+/-	+	+	+	+	+	+	+/-
MLY0124	+	+	+	+	+	+	+	+
MLY0126	+	+	+	+	+	+	+	+
MLY0134	+	+	+	+	-	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0145	-	ND	+	+	+	+	+	+
MLY0209	+/-	+	+	+	-	+	+	+
MLY0304A	+/-	+	+	+	+	+	+	+
MLY0307	-	+	+	+	+	+	+	+
MLY0309A	+	-	+	+	+	+	+	+
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	+	-	+	+	+	+
MLY0402	+	+	+	+	-	+	+	+
MLY0416	+	+	+	+	+	-	+	+
MLY0512	+	+	+	+	+	+	+	+
MLY0513	+	+	+	+	+	+	+	-
MLY0522	+/-	+	+	+	+	+	+	+
MLY0524	+	+	+	+	+	+	+	+
MLY0526	+	+	+	+	+	+	+	+
MLY0527A	+	+	+	+	+	+	+	+
MLY0701	+	+	+	+	+	+	+	+
MLY0704	+	+	+	+/-	+	+	+	+
MLY0705	+	+	+	+	+	+	+	+
MLY0706	+	+	+	+	+	+	+	+
MLY0709	+	+	+	+	+	+	+	+
MLY0716	+/-	+	+	+	+	+	+	+
MLY0725	+	+	+	-	+	+	+	+
MLY0729	+	+	+	+	+	+	+	+
MLY0731	+	+	+	+	+	+	+	+
MLY0732	+	+	+	+	+	+	+	+
MLY1105	+	+	+	-	+	+	+	+
MLY1143	-	+	+	+	+	+	+	+
MLY1153	-	+	+	+	+	+	+	+
MLY1156	+	+	+	+	+	+	+	+
MLY1161	+	+	+	+	+	+	+	+
MLY1214	+	+	+	+	+	+	+	+
MLY1232	-	+	+	+	+	+	+	+
MLY1233	+	+	+	+	+	+	+	+
MLY1311	+	+	+	-	+	+	+	+
vps1	-	+	+	+	+	+	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	ND	+	+	+	+	+	+	+
vps34	+	+	+	+	+	+	+	+
D597-6D	ND	+	ND	ND	+	+	+	+

Table 4.4 - continued

α-mutant / **a**-mutant	MLY0522	MLY0524	MLY0526	MLY0527A	MLY0701	MLY0704	MLY0705	MLY0706
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	ND
MLY0104	+	+	+	+	+	+	+	+
MLY0106	-	+	+	+	+	+	+	+
MLY0108	+/-	+	+	+	+	+	+	+
MLY0111	+	+	+	+	+	+	+	+
MLY0117	+	ND	+	ND	+	+	+	+
MLY0120	+	+	+	+	+	+	+	+
MLY0124	+	+	+	+	+	+	+	+
MLY0126	+	+	+	+	+	+	+	+
MLY0134	+	+	+	+	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0145	-	+	+	+	+	+	+	+
MLY0209	+	+	+	+	+	+	+	+
MLY0304A	-	+	+	+	+	+	+	+
MLY0307	-	+	+	+	+	+	+	+
MLY0309A	+	+	+	+	+	+	+	+
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	+	+	+	-	+	-
MLY0402	+	+	+	+	+	+	+	+
MLY0416	+	+	+	+	+	+	+	+
MLY0512	+	+	+	+	+	+	+	+
MLY0513	+	+	+	+	+	+	+	+
MLY0522	+	+	+	+	+	+	+	+
MLY0524	-	-	+	+	+	-	+	-
MLY0526	+	+	-	+	+	+	+	+
MLY0527A	+	+	+	-	+	+	+	+
MLY0701	+	+	+	+	-	+	+	+
MLY0704	-	+	+	+	+	-	+	-
MLY0705	+	+	+	+	+	+	-	+
MLY0706	+	+	+	+	+	-	+	-
MLY0709	+	+	+	+	+	+	+	+
MLY0716	+	+	+	+	+	+	+	+
MLY0725	+	+	+	+	+	-	+	-
MLY0729	+	-	+	+	+	+	+	+
MLY0731	+	-	+	+	+	+	+	+
MLY0732	+	+	+	+	+	+	+	+
MLY1105	+/-	+	+	+	+	-	+	-
MLY1143	-	+	+	+	+	+	+	+
MLY1153	-	+	+	+	+	+	+	+/-
MLY1156	+	+	+	+	+	+	+	+
MLY1161	+	+	+	+	+	+	-	+
MLY1214	+	+	+	+	+	-	+	-
MLY1232	-	+	+	+	+	+	+	+
MLY1233	+	+	+	+	+	+	+	+
MLY1311	+	+	+	+	+	-	+	+
vps1	+/-	+	+	+	+	+	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	+	+	+	+	ND	+	ND
vps34	+	+	+	+	+	+	+	ND
D597-6D	ND	ND	+	+	+	ND	+	ND

Table 4.4 - continued

α-mutant / **a**-mutant	MLY0709	MLY0716	MLY0725	MLY0729	MLY0731	MLY0732	MLY1105	MLY1143
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	+	+	+	+	+	+	+	-
MLY0106	+	+	+	+	+	+	+	-
MLY0108	+	+	+	+	+	+	+	+/-
MLY0111	+	+	+	+	+	ND	+	+
MLY0117	ND	+	+	+	+	ND	+	+
MLY0120	+	+	+	+	+	+	+	+
MLY0124	+	ND	ND	ND	ND	+	+	+
MLY0126	+	+	+	+	+	+	+	+
MLY0134	+	ND	+	+	+	ND	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0145	+	+	+	+	+	+	+	-
MLY0209	+	+	+	+	+	+	+	+
MLY0304A	+	+	+	+	+	+	+	+
MLY0307	+	+	+	+	+	+	+	-
MLY0309A	+	+	+	+	+	+	+	+
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	-	+	+	+	-	+
MLY0402	+	+	+	+	+	+	+	+
MLY0416	+	+	+	+	+	+	+	+
MLY0512	+	+	+	+	+	+	+	+
MLY0513	+	+	+	+	+	+	+	+
MLY0522	+	+	+	+	+	+	+	+
MLY0524	+	+	+	-	-	+	+	+
MLY0526	+	+	+	+	+	+	+	+
MLY0527A	+	+	+	+	+	+	+	+
MLY0701	+	+	+	+	+	+	+	+
MLY0704	+	+	-	+	+	+	-	+
MLY0705	+	+	+	+	+	+	+	+
MLY0706	+	+	-	+	+	+	-	+
MLY0709	-	+	+	+	+	+	+	+
MLY0716	+	-	+	+	+	+	+	+
MLY0725	+	+	-	+	+	+	-	+
MLY0729	+	+	+	-	-	+	+	+
MLY0731	+	+	+	-	-	+	+	+
MLY0732	+	+	+	+	+	-	+	+
MLY1105	+	+	-	+	+	+	-	+
MLY1143	+	+	+	+	+	+	+/-	-
MLY1153	+	+	+	+	+	+	+	-
MLY1156	+	+	+	+	+	+	+	+
MLY1161	+	+	+	+	+	+	+	+
MLY1214	+	+	-	+	+	+	-	+
MLY1232	+	+	+	+	+	+	+	-
MLY1233	+	+	+	+	+	+	+	+
MLY1311	+	+	-	+	+	+	-	+
vps1	+	+	+	+	+	+	+	-
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	+	+	ND	+	+	+	ND	+
vps34	+	+	+	+	+	+	+	+
D597-6D	ND	ND	ND	+	+	+	+	ND

Table 4.4 - continued

α-mutant / **a**-mutant	MLY1153	MLY1156	MLY1161	MLY1214	MLY1232	MLY1233	MLY1311	*vps1*
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	+	+	+
MLY0104	-	+	+	+	-	+	+	+/-
MLY0106	-	+	+	+	-	+	+	-
MLY0108	+	+	+	+	+	+	+	+/-
MLY0111	+	ND	+	+	+	+	ND	+
MLY0117	+	ND	+	ND	+	+	ND	+
MLY0120	+	+	+	+	+	+	+	+/-
MLY0124	+	ND	ND	+	+	ND	ND	+
MLY0126	+	+	+	+	+	+	+	+
MLY0134	+	ND	ND	+	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0145	-	ND	+	+	-	+	ND	-
MLY0209	+	+	+	+	+	+	+	+
MLY0304A	-	+	+	+	-	+	+	-
MLY0307	-	+	+	+	-	+	+	-
MLY0309A	+	+	+	+	+	+	+	+
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	+	-	+	+	-	+
MLY0402	+	+	+	+	+	+	+	+
MLY0416	+	+	+	+	+	+	+	+
MLY0512	+	+	+	+	+	+	+	+
MLY0513	+	+	+	+	+	+	+	+
MLY0522	+	+	+	+	+	+	+	+
MLY0524	+	+	+	+	+	+	+	+
MLY0526	+	+	+	+	+	+	+	+
MLY0527A	+	+	+	+	+	+	+	+
MLY0701	+	+	+	+	+	+	+	+
MLY0704	+/-	+	+	-	+	+	-	+
MLY0705	+	+	-	+	+	+	+	+
MLY0706	+	+	+	-	+	+	-	+
MLY0709	+	+	+	+	+	+	+	+
MLY0716	+	+	+	+	+	+	+	+
MLY0725	+	+	+	-	+	+	-	+
MLY0729	+	+	+	+	+	+	+	+
MLY0731	+	+	+	+	+	+	+	+
MLY0732	+	+	+	+	+	+	+	+
MLY1105	+	+	+	-	+	+	-	+
MLY1143	-	+	+	+	-	+	+	+
MLY1153	-	+	+	+	-	+	+	+
MLY1156	+	-	+	+	+	+	+	+
MLY1161	+	+	-	+	+	+	+	+
MLY1214	+	+	+	-	+	+	-	+
MLY1232	-	+	+	+	-	+	+	+
MLY1233	+	+	+	+	+	-	+	+
MLY1311	+	+	+	-	+	+	-	+
vps1	-	+	+	+	+	-	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	+	+	+	+	+	+	+
vps16	+	+	+	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	+	+	+	+
vps33	ND	+	+	ND	ND	+	ND	ND
vps34	+	+	+	+	+	+	+	+
D597-6D	ND	+	+	ND	+	+	ND	+

Table 4.4 - continued

α-mutant	*vps5*	*vps11*	*vps16*	*vps17*	*vps18*	*vps33*	*vps34*	B-4164
a-mutant								
SEY6211	+	+	+	+	+	+	+	+
MLY0101	+	+	+	+	+	+	+	+
MLY0103	+	+	+	+	+	ND	+	+
MLY0104	+	+	+	+	+	+	+	+
MLY0106	+	+	+	+	+	ND	+	+
MLY0108	+	+	+	+	+	+	+	+
MLY0111	+	+	+	+	+	ND	+	+
MLY0117	+	ND	ND	+	+	ND	+	+
MLY0120	+	+	+	+	+	+	+	+
MLY0124	+	+	+	+	+	+	+	+
MLY0126	+	+	+	+	+	+	+	+
MLY0134	+	+	+	+	+	+	+	+
MLY0139	+	+	+	+	+	+	+	+
MLY0145	+	+	+	+	+	ND	+	+
MLY0209	+	+	+	+	+	+	+	+
MLY0304A	+	+	+	+	+	+	+	+
MLY0307	+	+	+	+	+	ND	+	+
MLY0309A	+	+	+	+	+	+	+	+
MLY0309B	+	+	+	+	+	+	+	+
MLY0310	+	+	+	+	+	+	+	+
MLY0402	+	+	+	+	+	+	+	+
MLY0416	+	+	+	+	+	+	+	+
MLY0512	+	+	+	+	+	+	+	+
MLY0513	+	+	+	+	+	+	+	+
MLY0522	+	+	+	+	+	+	+	+
MLY0524	+	+	+	+	+	+	+	+
MLY0526	+	+	+	+	+	+	+	+
MLY0527A	+	+	+	+	+	+	+	+
MLY0701	+	+	+	+	+	ND	+	+
MLY0704	+	+	+	+	+	ND	+	+
MLY0705	+	+	+	+	+	+	+	+
MLY0706	+	+	+	+	+	ND	+	+
MLY0709	+	+	+	+	+	ND	+	+
MLY0716	+	+	+	+	+	+	+	-
MLY0725	+	+	+	+	+	+	+	+
MLY0729	+	+	+	+	+	+	+	+
MLY0731	+	+	+	+	+	+	+	+
MLY0732	+	+	+	+	+	+	+	+
MLY1105	+	+	+	+	+	ND	+	+
MLY1143	+	+	+	+	+	+	+	+
MLY1153	+	+	+	+	+	+	+	+
MLY1156	+	+	+	+	+	+	+	+
MLY1161	+	+	+	+	+	+	+	+
MLY1214	+	+	+	+	+	ND	+	+
MLY1232	+	+	+	+	+	ND	+	+
MLY1233	+	+	+	+	+	+	+	+
MLY1311	+	+	+	+	+	ND	+	+
vps1	+	+	+	+	+	ND	+	+
vps5	+	+	+	+	+	+	+	+
vps11	+	ND	ND	+	+	ND	+	+
vps16	+	+	-	+	+	+	+	+
vps17	+	+	+	+	+	+	+	+
vps18	+	+	+	+	-	+	+	+
vps33	+	+	+	+	ND	ND	+	+
vps34	+	+	+	+	+	+	-	+
D597-6D	+	+	ND	+	ND	ND	+	-

Table 4.4: Complementation of the osmosensitive growth phenotype of random spore mutant isolates. Every mutant of **a**-mating type was crossed against every mutant of α-mating type in rich liquid medium (YPD), and putative diploids were dotted onto YPD supplemented with 1.5 M NaCl, and growth or non-growth under selective conditions was scored after 14 days incubation at 30°C. Two mutant strains were said to complement if the putative diploid was able to form colonies and maintain growth under selective conditions (+). Two mutants were said not to complement if diploids (judged by their ability to sporulate) failed to grow at all under the selective conditions (-). Some diploid strains showed an intermediate phenotype in that they grew slowly but not as well as a homozygous wild type diploid strain (+/-). Certain other mutants failed to form diploids in the drop overlay complementation test and their complementation pattern could not be tested (ND = no diploids). The scores recorded in the table are the results of a single complementation test, and generally in good agreement with the previous complementation test (Table 4.3). Strains that failed to complement the NaCl sensitive phenotype of other strains were placed in one complementation group. Whenever possible, once back-cossed mutant strains with the same auxotrophy markers as the original mutant strain were used in subsequent experiments to reduce the risk of a synergistic phenotype due to several unrelated mutations

Complementation Group	Allele
*ssv*1	-1 (MLY0104), -2 (MLY0106), -3 (MLY0108), -4 (MLY0120), -5 (MLY0145), -6(MLY0304A), -7 (MLY0307), -8 (MLY1143), -9 (MLY1153), -10 (MLY1232), -11 (MLY1309), -12 (MLY1315)
*ssv*2	-1 (MLY0117), -2 (MLY0111)
*ssv*3	-1 (MLY0732)
*ssv*4	-1 (MLY0126)
*ssv*5	-1 (MLY0524), -2 (MLY0729), -3 (MLY0731)
*ssv*6	-1 (MLY0527A)
*ssv*7	-1 (MLY309A), -2 (MLY1005)
*ssv*8	-1 (MLY0134), -2 (MLY0209), -3 (MLY0402)
*ssv*9	-1 (MLY0310), -2 (MLY0704), -3 (MLY0706), -4 (MLY0725), -5 (MLY1105), -6 (MLY1214), -7 (MLY1311)
*ssv*10	-1 (MLY0103)
*ssv*11	-1 (MLY0124)
*ssv*12	-1 (MLY0139)
*ssv*13	-1 (MLY0526)
*ssv*14	-1 (MLY0701)
*ssv*15	-1 (MLY1502)
*ssv*16	-1 (MLY1156)
ssv (*ssv*17)	-100 (MLY0419), -101 (MLY0508), -102 (MLY0601), -103 (MLY0728), -104 (MLY0803), -105 (MLY0805), -106 (MLY0806), -107 (MLY0808), -108 (MLY0810), -109 (MLY0812), -110 (MLY0816), -111 (MLY0902), -112 (MLY0905), -113 (MLY0908), -114 (MLY0913), -115 (MLY1124), -116 (MLY1141), -117 (MLY1162), -118 (MLY1164), -119 (MLY1176), -120 (MLY1203), -121 (MLY1218), -122 (MLY1219)
*ssv*18	-1 (MLY0416)
*ssv*19	-1 (MLY0709)

Complementation Group	Allele
*ssv*20	-1 (MLY1161)
ssv	-123 (MLY0202), -124 (MLY0712)
*osm*3	-1 (MLY0513)
*osm*4	-1 (MLY0705)

Table 4.5: *ssv* and *osm* complementation groups. Two mutations were said to complement each other, if heterozygous diploids were able to grow on YPD + 1.5 M NaCl after 7 days (see text). The validity of this complementation group was confirmed by others with the in the text mentioned three exceptions. The assignment of mutants into complementaion groups was based solely by complementation of the osmosensitive phenotype. The assignment of mutants in *ssv17* is preliminary and based on their similar semi-dominant phenotype rather than true complementation of alleles in this group. They are fully complemented by the wild type. *osm3* and *osm4* are complementation groups of two mutants that have no other defects except in maintainig growth on YPD + 1.5 M NaCl. The two *ssv*-123 and *ssv*-124 alleles are sterile, and their normal complementation pattern could not be assessed. They may or may not be identical. One mutant of each complementation group having more than one member was chosen to represent the complementation group in subsequent experiments. These "type alleles", namely *ssv1*-8, *ssv5*-2, *ssv7*-1, *ssv8*-2, *ssv9*-7 and *ssv*-100, were chosen because their *vps* phenotype as well as vacuolar morphology defect was clearly distinctive from the wild type (see below).

Strain	Parent	Genotype
MLY1501	MLY0103	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ssv10*-1
MLY1502	MLY0716	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ssv-aro7*
MLY1503	MLY0310	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ts ssv9*-1
MLY1505	MLY0526	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ssv13*-1
MLY1506	MLY0304	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ssv1*-6
MLY1507	MLY0111	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *ade2*-101 *suc2*-Δ9 *GAL ssv2*-2
MLY1508	MLY0304	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ssv1*-7
MLY1509	MLY1143	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *suc2*-Δ9 *GAL ssv1*-8
MLY1510	MLY0527A	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *suc2*-Δ9 *GAL ssv6*-1
MLY1511	MLY0309	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *ade2*-101 *suc2*-Δ9 *GAL ssv7*-1
MLY1512	MLY0527A	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *suc2*-Δ9 *GAL ssv6*-1
MLY1513	MLY0732	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ts ssv3*-1
MLY1514	MLY0732	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *ade2*-101 *suc2*-Δ9 *GAL ts ssv3*-1
MLY1515	MLY0729	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *ade2*-101 *suc2*-Δ9 *GAL ssv5*-2

Strain	Parent	Genotype
MLY1516	MLY0729	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *ade2*-101 *suc2*-Δ9 *GAL ssv5*-2
MLY1517	MLY0309	*MAT***a** *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *ade2*-101 *suc2*-Δ9 *GAL ssv7*-1
MLY1518	MLY1311	*MAT*α *leu2*-3,112 *ura3*-Δ52 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *ade2*-101 *suc2*-Δ9 *GAL ssv9*-7

Table 4.6: Strain designation and phenotype of random spore isolates from heteozygous diploids between osmosensitive mutant strains and their isogenic wild type. The auxotrophy phenotypes and mating type of each strain were tested. Unless indicated otherwise, these strains rather than the originally isolated mutant alleles were used in subsequent experiments.

4.2.2. Vacuolar Morphological Analysis

4.2.2.1. Light and Fluorescence Microscopy

Light microscopic analysis, using Nomarski contrast optics, of all 97 originally isolated mutants showed that a large proportion had an altered vacuolar morphology (Table 4.7). One group had many small vacuole like structures, whereas another group did not show any vacuoles at all. These findings are consistent with previous findings (Banta *et al.*, 1988), which led to the classification of *vps* mutants into three discrete vacuolar morphology classes: Class A with large, normal appearing vacuoles, Class B with many small vacuoles, and Class C with no detectable vacuoles. Vital staining, using FITC, quinacrine or CDCFDA were initially attempted in this study, but it yielded somewhat ambiguous results, because an adequate fluorescence microscope was not

available at the time. Thus only the vacuolar appearance under Normarski contrast microscopy was recorded.

4.2.2.2. Indirect Immunofluorescence Microscopy

Alhough the morphological characterization of the *ssv* mutants isolated in this study was facilitated by electron microscopy, a rapid method that relatively unambiguously could identify vacuolar morphology in a short time period was sought. The immunolocalization of two vacuolar membrane markers, Pho8p and the 60 kDa subunit of the vacuolar ATPase could give a valid estimate of the morphology of the vacuole (Raymond *et al.*, 1991). This method was used to assess the vacuolar morphology of mutants that escaped classification by electron microscopy (Table 4.7).

4.2.2.3. Electron Microscopy

The results obtained by light microscopy and fluorescence microscopy were verified by electron microscopy, using a preparation method specifically staining double-membranes and carbohydrate structures that accentuates vacuoles (Table 4.7). Class A mutants had one to three large, dark staining vacuoles. Class B mutants had between 4 to 15 small, disrupted vacuole-like structures per thin-section and cell. All other mutants, which had no normal staining or no vacuoles at all, were said to be class C (Figure 4.2). One striking feature was that not all alleles of one complementation group had an identical vacuolar morphology. These findings are consistent with previous observations (Klionsky *et al.*, 1990; S. Emr, personal communication), where different *vps* alleles of the same complementation group have different vacuolar morphologies. Thus for a gene to be placed in class A, all mutant alleles must be of this phenotype. Individual mutants in a class B gene may exhibit either A or B morphology, while mutants in class C may exhibit all three morphologies. Taken together with the complementation data previously obtained, these considerations allowed the classification of the complementation groups in terms of vacuolar morphology (Table 4.8). Eight groups contain alleles all of which show class A morphology (*ssv4*, *ssv10*,

ssv11, *ssv12*, *ssv14*, *ssv20*, *osm3* and *osm4*). Twelve groups contain alleles, which are either of class B or class A morphology (*ssv2*, *ssv5*, *ssv6*, *ssv7*, *ssv8*, *ssv13*, *ssv16*, *ssv18*, *ssv19*, *ssv*-123 and *ssv*-124). Five groups had alleles which were only class C (*ssv1*, *ssv3*, *ssv9*, *ssv15* and *ssv17*). Of the above classes, only class C is a final classification, because complementation groups of A or B morphology may reflect the failure to isolate alleles of C morphology.

A common feature of members of *ssv17* complementation group alleles was that some mutant alleles conferred a mixed vacuolar phenotype in that about 50% of all cells had vacuoles present while the remaining cells had a class C phenotype, indicative of a vacuolar segregation defect. A vacuolar segregation defect was detectable in alleles of several complementation groups (*ssv2*, *ssv5*, *ssv8*, *ssv9* and *ssv17*). Its characteristic morphological appearance was that some cells were class A while others were class C. In all cases large mother cells had a clearly visible vacuole, while daughter cells of the first generation were devoid of any detectable vacuolar compartment. This phenotype was scored as A/C in Table 4.7.

Table 4.7: Vacuolar morphology

Strain	Vacuolar Morphology by				
	Normarski	Qinacrine	Pho8p IIF	60 k IIF	EM
SEY6210	A	+	A	A	A
SEY6211	A	+	ND	ND	ND
15113-8	A	+	ND	ND	A
14121-26	B	+/-	ND	ND	B
0552-11	C	+/-	ND	ND	C
MLY0101	C	-	A/C	A/C	C
MLY0103	B/C	-	A	A	A
MLY0104	A/B	+/-	B	B	B
MLY0106	A/B	+/-	ND	ND	B
MLY0107	A	+	ND	ND	ND
MLY0108	B	+/-	ND	ND	ND
MLY0109	A/B	+/-	ND	ND	B
MLY0111	B	+	B	B	B
MLY0113	B^{lv}	+/-	ND	ND	B
MLY0115	A	+	ND	ND	A
MLY0116	B	+	ND	ND	ND
MLY0117	A^{r}/C	+	A	A	B
MLY0120	B/C	+/-	ND	ND	B
MLY0121	C	+/-	ND	ND	A
MLY0124	A/C	+	A/C	A/C	A/C

Strain	Vacuolar Morphology by				
	Normarski	Qinacrine	Pho8p IIF	60 k IIF	EM
MLY0125	A/B	+	ND	ND	ND
MLY0126	A/B	+	ND	ND	A/B
MLY0127	A/B	+/-	ND	ND	A/B
MLY0134	B	+/-	ND	ND	B
MLY0139	A	+/-	ND	ND	ND
MLY0144	A	-	ND	ND	A/B
MLY0145	C	ND	ND	ND	A/B
MLY0202	A/B	ND	ND	ND	B
MLY0209	B	ND	ND	ND	B
MLY0304	B/C	ND	ND	ND	A
MLY0307	B	ND	B	B	ND
MLY0309	A/B	ND	ND	ND	ND
MLY0310	$A^{r/lc}$	ND	C	C	Abnormal
MLY0402	A/B	ND	A	A	ND
MLY0416	B/C	ND	ND	ND	B
MLY0419	B/C	ND	C	B/C	B/C
MLY0420	A/C	ND	ND	ND	ND
MLY0508	A/C	ND	A/C	A/C	C
MLY0512	B/C	ND	ND	ND	B
MLY0513	C	ND	ND	ND	A
MLY0522	B/C	ND	ND	ND	B
MLY0524	A/B/C	ND	ND	ND	A
MLY0526	A^{l}	ND	A	A	B
MLY0527	A	ND	ND	ND	A
MLY0601	B/C	ND	B/C	B/C	ND
MLY0701	A/B	ND	ND	ND	A
MLY0702	A/C	ND	ND	ND	A
MLY0704	C	ND	A/C	A/C	C
MLY0705	C	ND	ND	ND	A
MLY0706	B/C	ND	ND	ND	A/C
MLY0709	C	ND	ND	ND	B
MLY0712	A/B	ND	ND	ND	ND
MLY0713	B	ND	ND	ND	A
MLY0716	C	ND	ND	ND	B
MLY0725	C	ND	A	A	A
MLY0728	B/C	ND	ND	ND	B
MLY0729	C	ND	ND	ND	B
MLY0731	A/C	ND	ND	ND	ND
MLY0732	C	ND	ND	ND	ND
MLY0803	B	ND	ND	ND	A/B
MLY0805	C	ND	B	B	B
MLY0806	A	ND	A	A	ND
MLY0808	A	ND	ND	ND	A
MLY0810	A	ND	ND	ND	A/B
MLY0811	A	ND	ND	ND	ND
MLY0812	B	ND	B	B	B
MLY0813	A/B	ND	ND	ND	B/C
MLY0815	C	ND	ND	ND	ND
MLY0816	A	ND	A	A	ND
MLY0902	A	ND	A	A	B
MLY0905	A	ND	A	A	B
MLY0908	A	ND	A	A	ND
MLY0911	A	ND	ND	ND	B
MLY0913	B	ND	ND	ND	B
MLY1001	A/B	ND	ND	ND	ND
MLY1002	C/B	ND	ND	ND	A
MLY1005	A	ND	ND	ND	B

Strain	Vacuolar Morphology by				
	Normarski	Qinacrine	Pho8p IIF	60 k IIF	EM
MLY1006	C	ND	ND	ND	A
MLY1105	B	ND	ND	ND	C
MLY1124	B	ND	ND	ND	ND
MLY1129	C	ND	ND	ND	A
MLY1138	C	ND	ND	ND	A/B
MLY1141	B/C	ND	ND	ND	ND
MLY1143	C	ND	B	B	B
MLY1153	A/B	ND	A/C	A/C	C
MLY1156	B	ND	ND	ND	B
MLY1161	C	ND	ND	ND	A/B
MLY1162	C	ND	ND	ND	B
MLY1164	A	ND	ND	ND	ND
MLY1176	C	ND	ND	ND	A
MLY1203	B/C	ND	ND	ND	A
MLY1214	B	ND	A	A	AR
MLY1218	B/C	ND	ND	ND	B
MLY1219	C	ND	ND	ND	ND
MLY1232	C	ND	ND	ND	A/B
MLY1233	C	ND	ND	ND	A
MLY1309	A	ND	ND	ND	A/B
MLY1311	B/C	ND	C	C	C
MLY1315	C	ND	A	A	A/B
MLY1330	A	ND	ND	ND	A
MLY1332	B	ND	ND	ND	ND
MLY1511		ND	ND	ND	A/B

Table 4.7: Vacuole morphology of mutant alleles. The data compiled in this table were obtained from examining at least two independent cultures of the mutant strains for each method. The criteria for assignment of vacuolar classes are described in the text. A: class A vacuolar morphology; B: class B vacuolar morphology; C: class C vacuolar morphology; A/C: vacuolar segregation defect; B/C: mixed poulation of class B and class C vacuolar phenotype; l: large vacuoles; lv: large vacuoles surrounded by vesicles; r: rough vacuolar membrane; ND: not determined.

Among a small selection of mutants screened for a vacuolar acidification defect using quinacrine as a vacuole specific fluorophore, two mutants, MLY0103 (*ssv10*-1) and MLY0144, with apparently normal morphological vacuoles failed to show quinacrine fluorescence indicative for a vacuolar acidification defect. The *ssv10*-1 allele did not have defects associated with vacuolar membrane ATPase mutants such as sensitivity to Ca^{2+} or a Pet^- phenotype (see later sections). MLY0144 could not be

assigned to any complementation group. Several mutants screened for the proper assembly of the vacuolar ATPase by determining the localization of the 60 kDa vacuolar ATPase subunit to the vacuolar membrane did not have any aberrant assembly compared to Pho8p as a vacuolar membrane marker. There was an obvious discrepancy between the results obtained by light microscopy and electron microscopy. Assuming that not all vacuolar structures could be seen by light microscopy or that some vacuoles might have an altered refractive index making them invisible to Normarski contrast microscopy, only results obtained by electron microscopy were used for classification purposes.

SSV	Total Alleles	*ts* Alleles	Vacuolar Morphology			Vacuolar Class
			Class A	Class B	Class C	
1	12	0	5	6	1	C
2	2	0	1	1	0	B
3	1	1	0	0	1	C
4	1	0	1	0	0	A
5	3	0	1	2	0	B
6	1	0	0	1	0	B
7	2	0	1	1	0	B
8	3	0	0	3	0	B
9	8	1	3	0	5	C
10	1	0	1	0	0	A
11	1	0	1	0	0	A
12	1	0	1	0	0	A
13	1	0	0	1	0	B
14	1	0	1	0	0	A
15	2	0	0	0	2	C
16	1	0	0	1	0	B
17	23	0	5	16	2	C
18	1	0	0	1	0	B
19	1	0	0	1	0	B
20	1	0	0	1	0	B
Sterile	2	0	0	2	0	B

Table 4.8: Classification of *ssv* complementation groups according to vacuolar morphology. A: normal intact vacuoles; B: small disrupted vacuoles; C: no vacuoles (see text).

4.2.3. Morphological Features

Apart from differences in vacuolar morphology, some mutants had aberrant morphological phenotypes compared to the wild type (Figure 4.3). Excessively large

vacuoles were observed in strains MLY0127, MLY0310 (*ssv9*-1) and MLY0526 (*ssv13*-1) (Figure 4.3 B-D). A similar phenotype has been described before, observable in two *vps* mutant strains (Banta *et al.*, 1988). It has been suggested that these giant vacuoles could have a osmoregulatory defect. Complementation analysis (Tables 4.3 and 4.5) provides evidence that these mutant alleles are not identical to prevoiously isolated *vps* mutants exhibiting a similar phenotype where viable diploids could be generated. MLY0127 cells are not spherical but "flattened", suggesting another defect in cell wall morphogenesis. MLY0127 could not unambiguously be assigned to any complementation group. Budding defects were associated with two mutant strains, MLY0124 (*ssv11*-1) and MLY0704 (*ssv9*-2). Other observable defects included vacuoles surrounded by larger vesicles in MLY0113, an increase in overall cell size in MLY0116, large, "shmoo"-like cells in MLY0120 (*ssv1*-4), small cells of variable shape in MLY0144 and the formation of large cell aggregates in culture that led to the formation of pellets in MLY0134 (*ssv8*-1). Various aberrant subcellular structures were also detectable in some alleles of *ssv1*, *ssv2*, *ssv5*, *ssv7*, *ssv8*, *ssv9*, *ssv10*, *ssv*-121 and *osm3* (Figure 4.4), which showed either similarity to previously identified Berkeley-bodies and Golgi-like organelles (Novick *et al.*, 1981) or organelles which stained like vacuoles in their periphery but whose center was electron transparent. These organelles appear to be taken up by vacuoles by a process similar to endocytosis and accumulate in some vacuolar class C mutants. The organelles were also found in the vacuole of mutant strains with an electron transparent vacuole probably due to leakage of the contents before or during fixation (Figure 4.4 H and M). In one strain these organelles underwent fusion or fission (Figure 4.4 P). These organelles are also present in some but not all class B cells and could represent intermediates in class B vacuole formation, especially since their density varied from completely transparent to completely dense (Figure 4.4 I). Some *ssv9*-7 class C mutant cells (10% of total) accumulate electron transparent membrane enclosed structures (Figure 4.4 L). These structures could represent lipid bodies or intermediates in vacuole formation, although they could not be found in wild type cells. Other alleles, as for example *ssv1*-10 accumulated structures with similarity to autophagic vacuoles (Figure 4.4 4), which in plants were identified to be involved in

cellular degradation processes (Dunn, 1990). Also, several cells of the *ssv5*-1, *ssv10*-1, *osm3*-1 alleles and strain MLY1138 did have cytoplasmic inclusions and organelles inside the vacuole, suggesting a role for the yeast vacuole in organelle degradation. Two alleles of *ssv17*, namely *ssv*-119 and *ssv*-121, had a mixed vacuolar phenotype in that cells had class A, class B and class C vacuoles, although they were repeatedly purified to ensure that the phenotype is not due to contamination by other strains with different vacuolar morphology. Other abnormalities found were tubular structures ("y"-bodies) in *ssv8*-2, an extreme class B phenotype in that the whole cytoplasm contained fragmented vacuoles in MLY0109, electron dense, crystal like inclusions ("x"-bodies) in *ssv11*-1 and extremely large vacuoles in MLY1006. The *osm3*-1 allele did have normal size vacuoles and did not secrete vacuolar proteins (see later sections). However, most vacuoles in the strain had larger transparent inclusions inside the vacuole. Similar inclusions have been suggested to represent polyphosphate deposits (Banta *et al.*, 1988). Since these inclusions are much smaller in wild type cells, the polyphosphate is probably deposited in this mutant but fails to be degraded. Since polyphosphates represent a charge balancing agent in a non-osmotically active form (see above), the putative accumulation of polyphosphates might be accompanied by an increase of cations in the vacuole which might have an adverse effect on osmoregulation of the cell. Thus *osm3*-1 might after all represent a *ssv* mutant in that its osmosensitivity is caused by a vacuolar defect. This mutant was not further analyzed. The nature of all organelles found to accumulate in some *ssv* mutants is not known. They might reflect aberrant structures resulting from a defect in vacuole formation and are not normally found in wild type yeast, except the "e"-bodies perhaps. Alternatively they might represent intermediates in vacuole formation whose biosynthesis pathway is delayed by some *ssv* mutations. Also, whether the engulfing of cytoplasmic material occurs in the wild type is not known. This process might play a role in the general starvation induced turn-over of cytoplasmic material, and some *ssv* mutants might simulate starvation conditions because of a defect in a biosynthetic pathway or signalling mechanism.

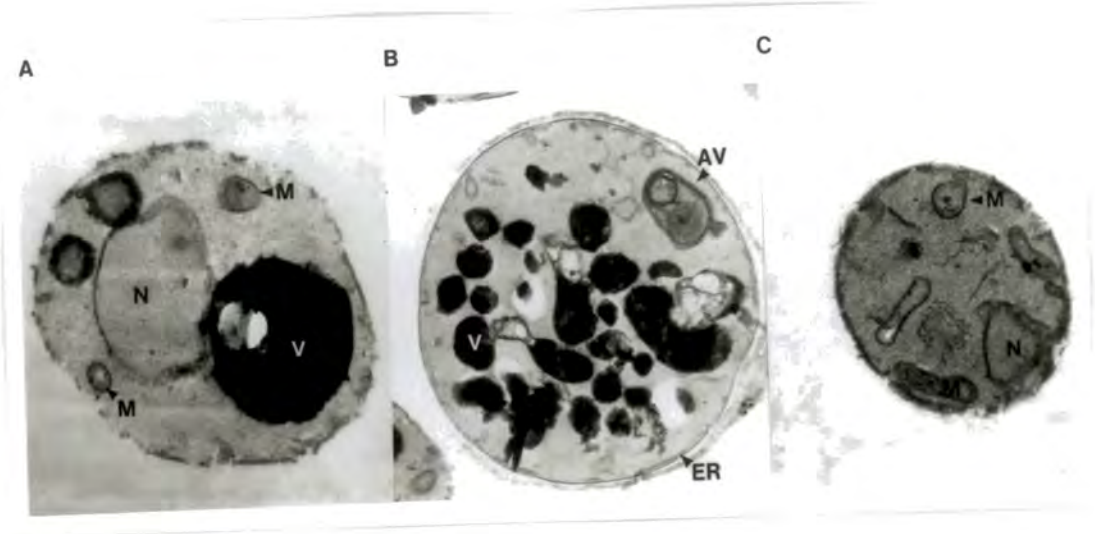


Figure 4.2: Electron microscopical classification of *ssv* mutants. Mutants that had one to three large vacuoles were of vacuolar class A (A; *ssv9*-6), mutants that had between 4 and 30 small vacuoles were of class B (B; *ssv1*-10), while mutats that did not have any vacuoles were of vacuolar class C (C; *ssv9*-7). The comparison between two alleles of the same complementation group (A and C) demonstrates that different alleles from one *ssv* complementation group can develop a different vacuolar morphology (see text). AV, autophagic vacuole; ER, endoplasmic reticulum; N, nucleus; M, mitochondrium; V, vacuole. Magnification: A, 19,500x; B, 19,500x; C, 19,500x.

Figure 4.3: Aberrant morphological featurs of *ssv* mutants as determined by Normarski contrast microscopy. Compared to the wild type SEY6210 (A), three strains, MLY0127 (B), MLY0310 (C) and to some degree MLY0526 (D) have enlarged vacuoles that take up a large proportion of the cytoplasm. MLY0126 (E) and MLY0704 (F) have a budding defect in that several buds form simultaneously. MLY0113 (G) has normal size vacuoles which are surrounded by samaller vesicles (see also Figure 4.4 J). MLY0116 (H) has large flattened cells. MLY0120 (I) has a shmoo-like appearance typical for cells that undergo zygote formation. However, fusion of these cells with each other could not be observed. MLY0144 (J) forms tube like structures possibly consisting of unseparated buds, while MLY0134 (K) forms large cell aggregates and flocculates in culture. 0.75 cm ≡ 1 μm.

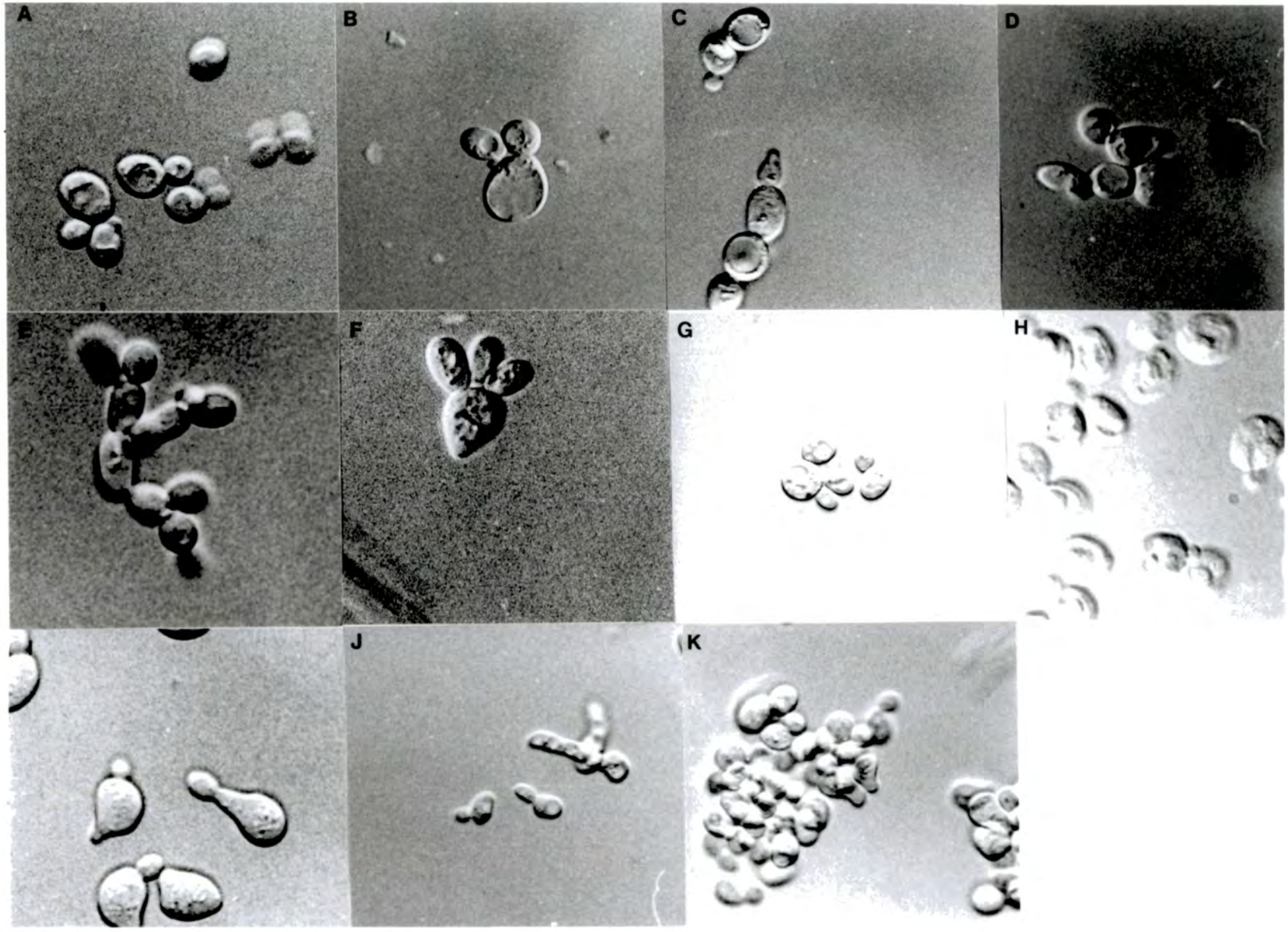

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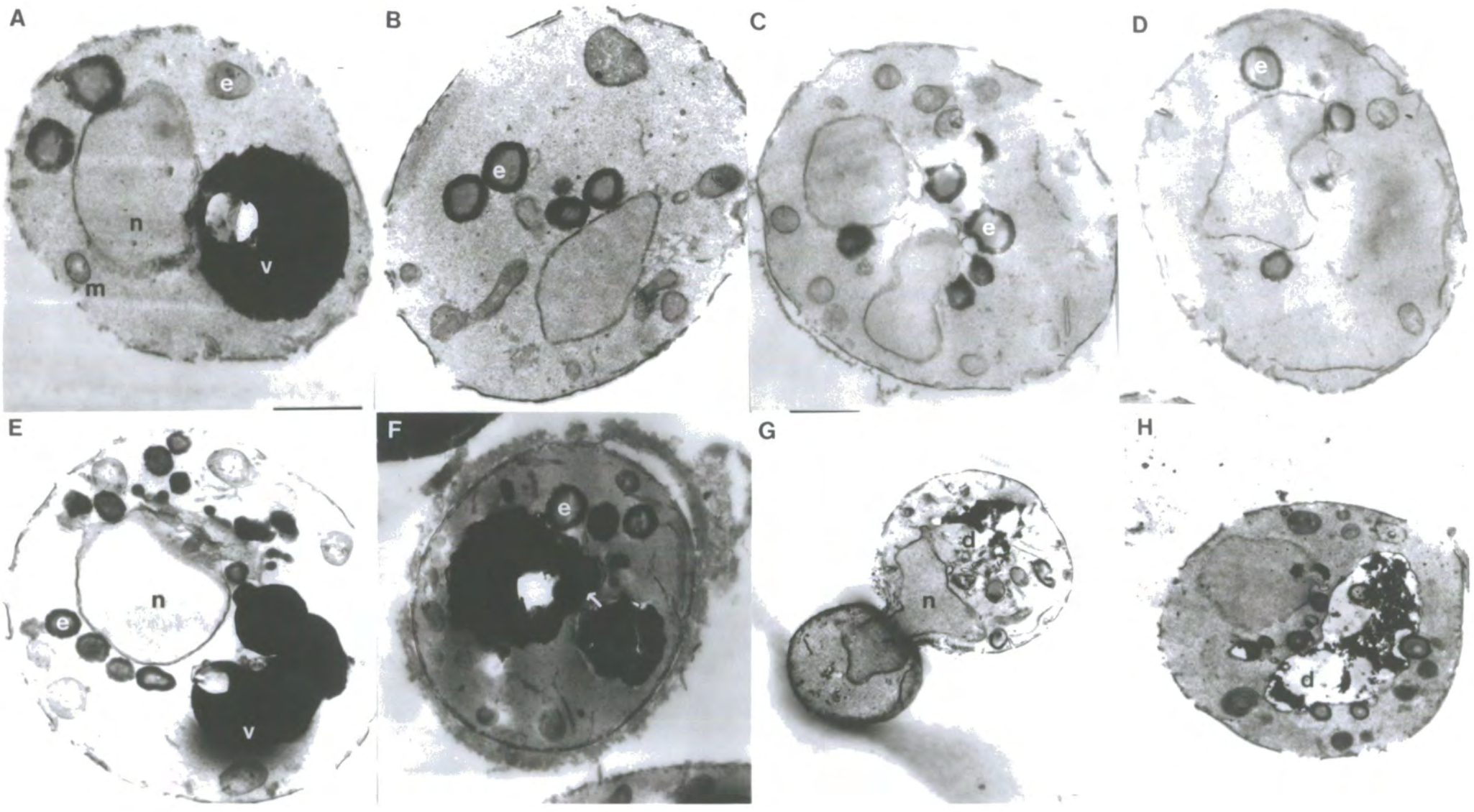
Figure 4.4: Ultrastructural examination of selected *ssv* mutants with atypical subcellular features. Strains MLY1214 *ssv9*-6 (A; 19,500x), MLY1005 *ssv7*-2 (B; 22,000x), MLY0101 (C, D; 19,500x), MLY0120 (E; 15,200x), MLY0117 *ssv2*-1 (F; 15,200x), MLY0121 (G; 7,000x), MLY0121 (H; 15,200x), MLY0716 (I; 19,500x), MLY0113 (J; 33,000x), MLY1311 *ssv9*-7 (K; 19,500x), MLY1311 *ssv9*-7 (L; 11,700x), MLY0111 *ssv2*-2 (M; 19,500x), MLY0103 *ssv10*-1 (N; 15,200x), MLY1233 (O; 15,200x), MLY1218 *ssv*-121 (P; 11,700x), MLY0524 *ssv5*-1 (Q; 15,200x), MLY1138 (R; 25,000x), MLY0103 *ssv10*-1 (S; 25,000x), MLY0134 *ssv8*-1 (T; 11,700x), MLY0101 (U; 19,500x), MLY0522 (V; 7,000x), MLY1218 *ssv*-121 (W; 11,700x), MLY1176 *ssv*-119 (Y; 7,000x), MLY0120 (Z; 15,200x), MLY0713 (1; 15,200x), MLY1002 *ssv7*-2 (2; 19,500x), MLY0117 *ssv2*-1 (3; 19,500x), MLY1232 *ssv1*-10 (4; 19,500x), MLY0513 *osm3*-1 (5; 15,200x), MLY1311 *ssv9*-7 (6; 117,000x), MLY0109 (7; 19,500x), MLY0209 *ssv8*-2 (8; 22,000x), MLY0109 (9; 14,000x), MLY0124 *ssv11*-1 (10; 29,500x) and MLY1006 (11; 22,000x) were prepared for electron microscopy as described in Materials and Methods. Some mutants accumulate organelles which probably represent prevacuolar compartments (e) (A-H, O, R, S). These organelles appear to be engulfed by the vacuole (F, arrow) and can be found in vacuoles that have lost part of its contents (M) or that are naturally electron transparent (d) (G, H). Some mutants are probably unable to take up these organelles which as a result accumulate around the vacuole (J). A small proportion of class C mutants accumulate membrane enclosed structures that have been previously suggested to be lipid bodies (l) (K, L) altough they also may represent aberrant vacuoles that do not accumulate glycoproteins. One strain (ssv10-1) has smaller "e" structures (p) (N), while others accumulate "e" structures that have a aberrant shape (O) or appear to fuse or divide (P). Vacuoles also are able to take up cytoplasmic material (Q-S), or to locate adjacent to the plasma membrane (T). Vacuoles in a normally class C strain (C, D) form in 5% of all buds and are sometimes surrounded by little vesicles (U). Mixed population phenotype was observed in some *ssv17* alleles (V-Y). Berkeley bodies (z) (Z-3), autophagic vacuoles (a) (4), Golgi structures (g) (6, 7) and tubules (y) (8) were also observable. Some mutants had disrupted (9) or intact (11) vacuoles that occupied an overproportional volume of the cell. Crystal-like structures (x) were detectable in *ssv11*-1 (10).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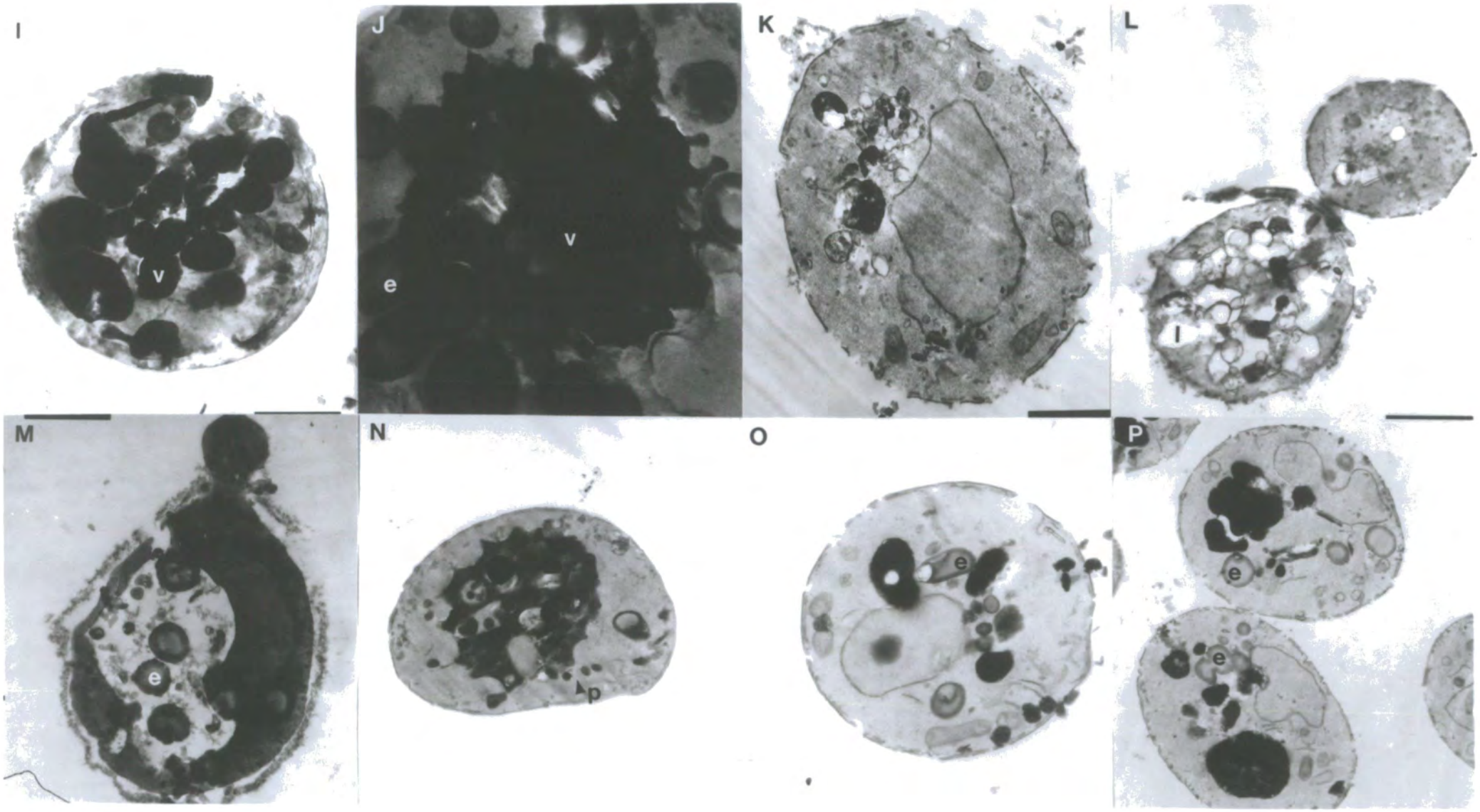
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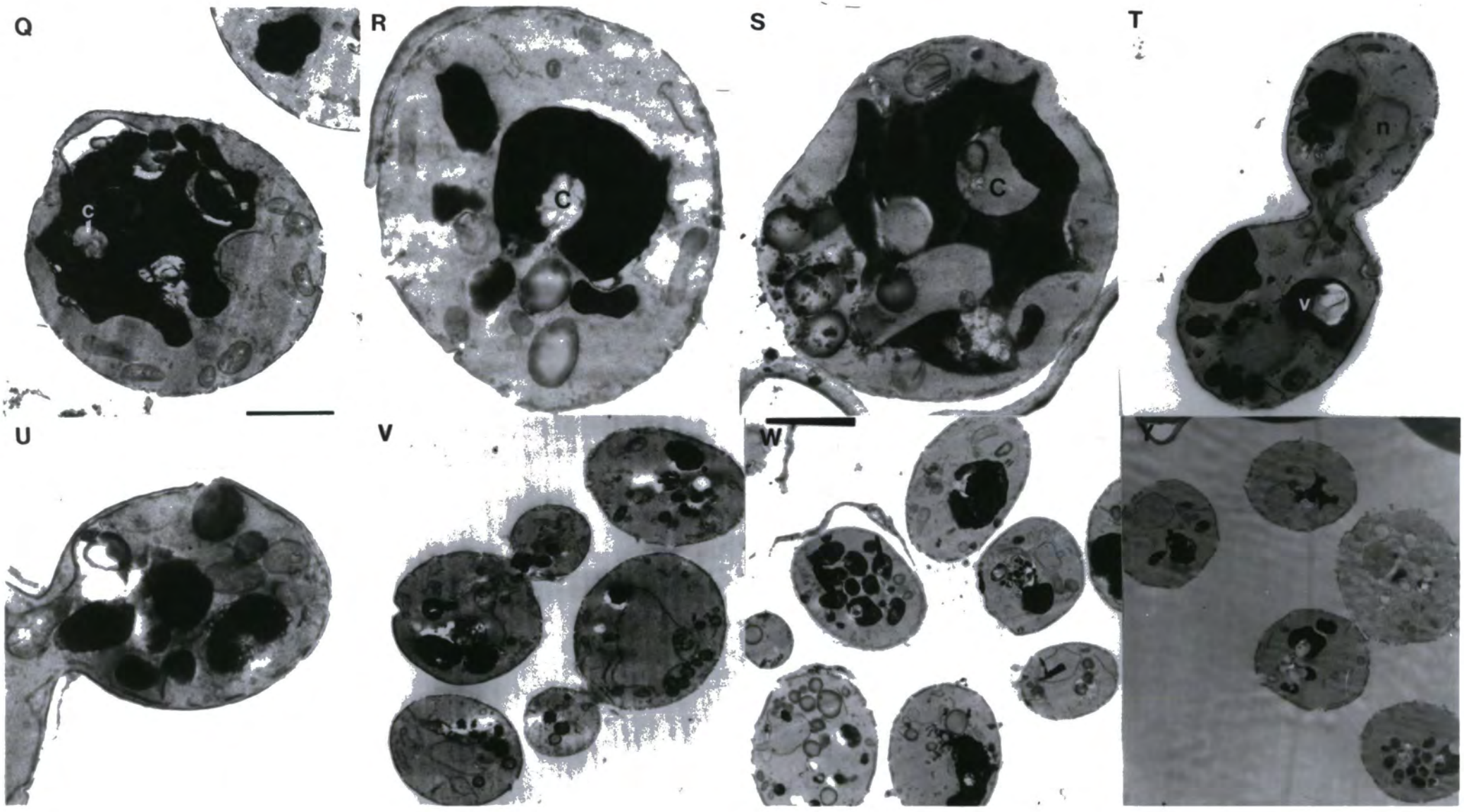
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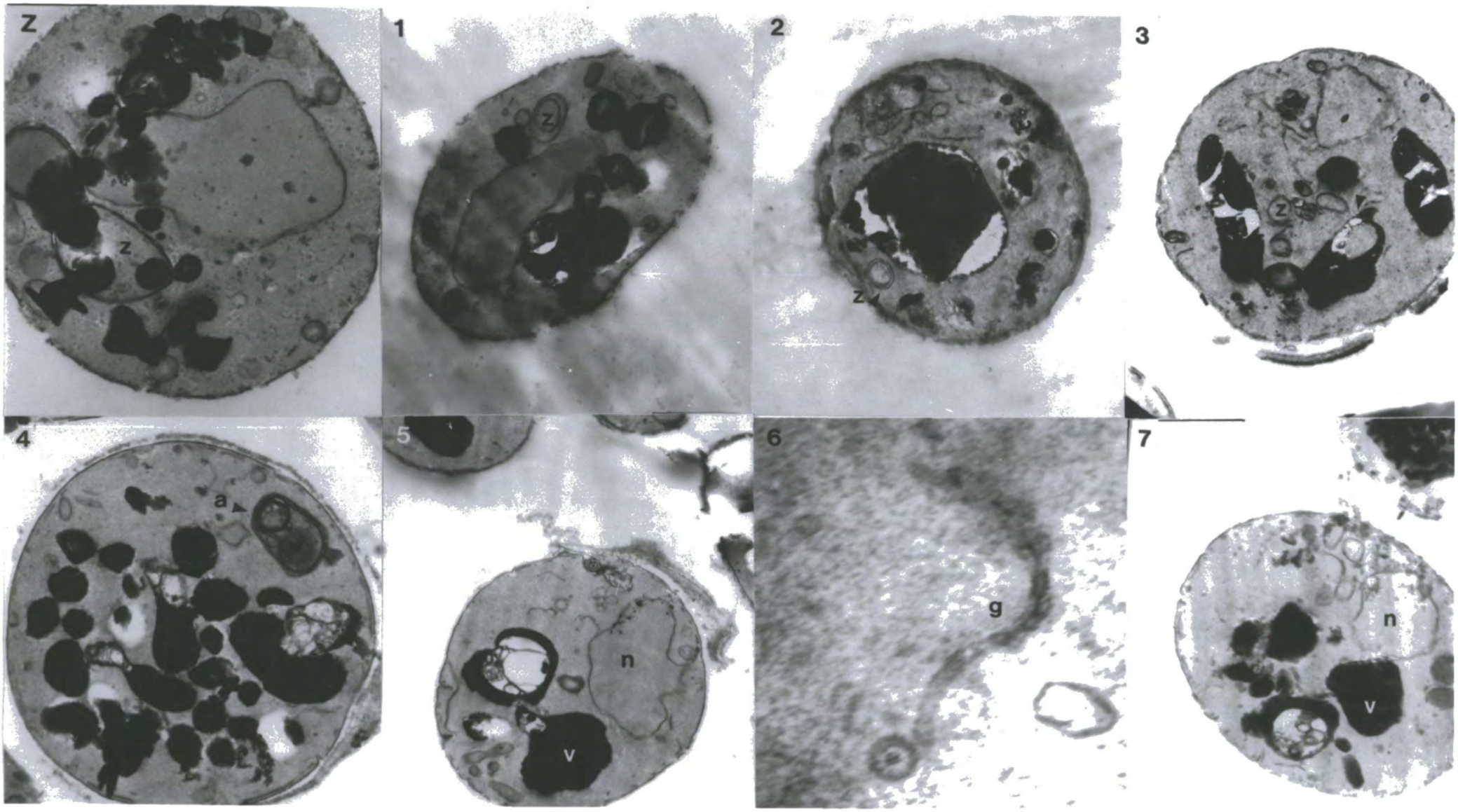
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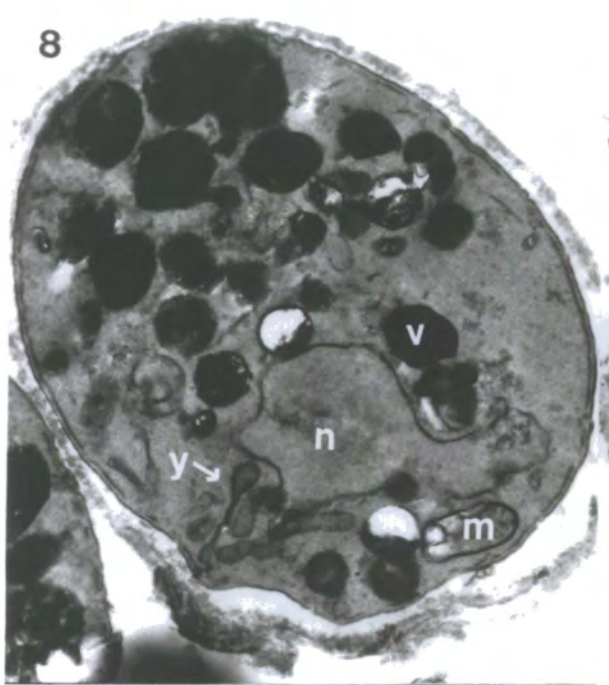
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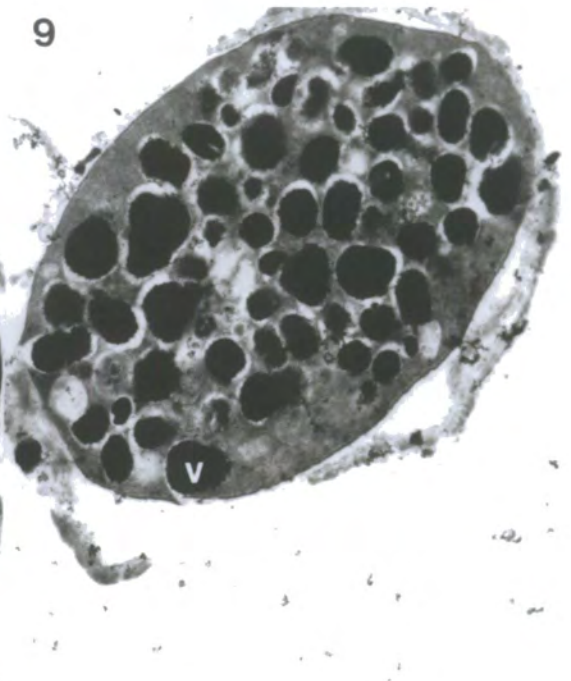
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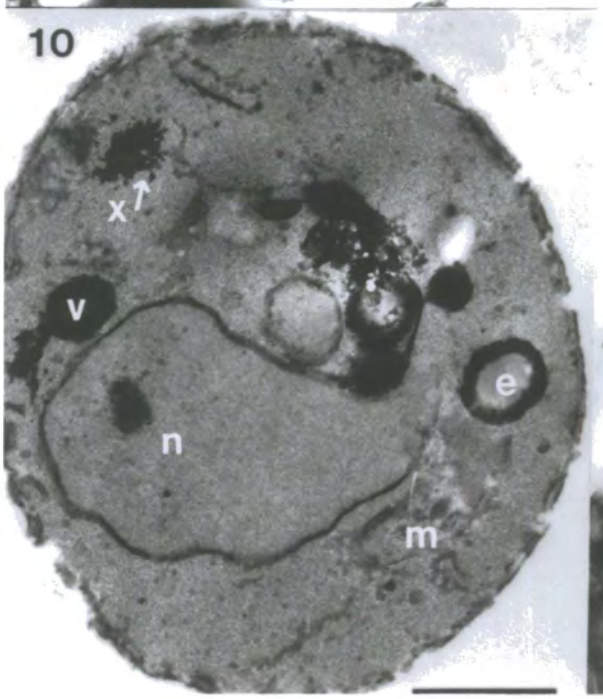
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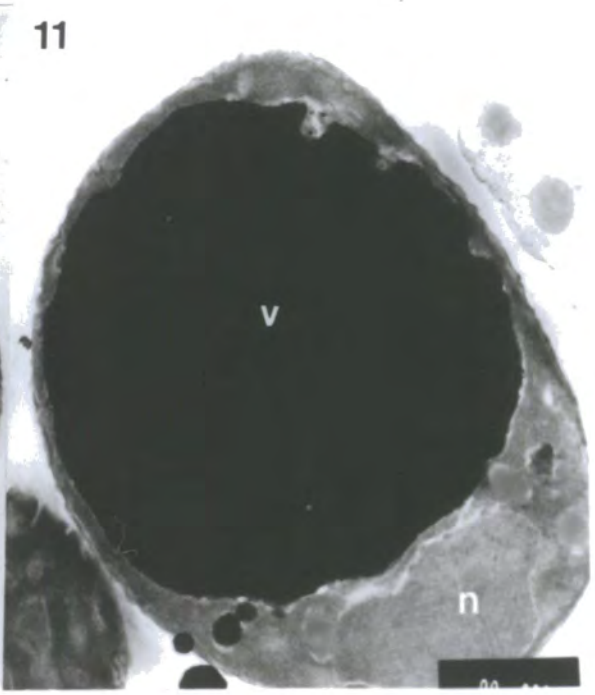
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4.2.4. Sensitivity to other high osmotic strength compounds

Since the mutants were selected on the basis of sensitivity to 1.5 M NaCl, they were subjected to plate assays to assess their ability to grow if other osmotically active components besides NaCl were added as a supplement to the medium. All mutants failed to grow on YPD medium containing 2.0 M sorbitol or 2.0 M glycerol, showing that they are truly osmosensitive and not selectively sensitive to Na^+ or Cl^- ions (Table 4.9). Only *ssv15*-1 showed weak growth if sorbitol or glycerol was the osmotically active substance. The fact that among the type alleles screened none had a selectively sensitive growth phenotype to NaCl or to other osmotically active components is potentially useful to interpret the nature of the mutants isolated in this study.

Type allele	Growth after 7 days on		
	1.5 M NaCl	2.0 M sorbitol	2.0 M glycerol
wt	+	+	+
ssv1-8	-	-	-
ssv2-1	-	-	-
ssv3-1	-	-	-
ssv4-1	-	-	-
ssv5-2	-	-	-
ssv6-1	-	-	-
ssv7-1	-	-	-
ssv8-2	-	-	-
ssv9-7	-	-	-
ssv10-1	-	-	-
ssv11-1	-	-	-
ssv12-1	-	-	-
ssv13-1	-	-	-
ssv14-1	-	-	-
ssv15-1	-	(-)	(-)
ssv16-1	-	-	-
ssv-100	-	-	-
ssv-123	-	-	-
ssv-124	-	-	-

Table 4.9: Osmosensitive phenotype of representative type alleles of all *ssv* complementation groups. The mutants were tested for their ability to grow on YPD supplemented with different osmotically active compounds. Except *ssv15*-1 which showed some growth on YPD supplemented with 2.0 M sorbitol or 2.0 M glycerol, all other *ssv* mutant alleles failed to grow on medium supplemented with different osmotically active compounds, unlike their isogeneic wild type.

4.2.5. Differential Growth Inhibition by NaCl and Sorbitol

The effect of two different osmosensitive growth inhibitors, NaCl and sorbitol, was tested on the wild type and a representative *ssv* mutant, *ssv1*-2. This study was initiated by the observation that the osmosensitive phenotype of all *ssv* mutants was absolute in that no growth was observed after 21 days on YPD + 1.5 M NaCl, while some growth could be detected on YPD agar supplemented with 2.5 M sorbitol after the same time and thus were still viable and able to grow. Two log-phase cultures of SEY6210 (wt) and MLY0106 (*ssv1*-2) were diluted to 2 x 10^6 cells/mL into YPD, YPD + 1.2 M sorbitol, YPD + 2.5 M sorbitol and YPD + 1.5 M NaCl. Growth was recorded at the start of the experiment immediately after inoculation (t=0) and after 24 hours (t=24) incubation at 30°C and 140 rpm by determining viable cell counts and total cell counts (Figure 4.5). While there is no obvious discrepancy in cell numbers between the wild type and the mutant grown in YPD after 24 hours, some growth inhibition occurs for both mutants in YPD + 1.2 M sorbitol. The growth of the *ssv1*-2 mutant is impeded at 2.5 M sorbitol, although growth itself is not completely inhibited. The use of 1.5 M NaCl as a supplement inhibits active growth of the mutant and severely impedes wild type growth. While the wild type total cell counts and the viable cell counts are in good agreement, no viable mutant cells could be recovered even at t=0 hours, strongly suggesting that 1.5 M NaCl is lethal to the mutant almost instantly (within approximately 10 seconds after inoculation after which a sample was taken), while sorbitol does not have such a drastic effect. These findings are indicative for a differential effect of osmotically active components on cell growth or survival. While in case of sorbitol the presence of the osmotically active compound alone is not sufficient for lethality, other osmotically active components are lethal if the strain carries a mutation in a gene confering osmosensitivity. This suggests the presence of an osmoregulatory mechanism in wild type yeast that facilitates the instant adaptation of the cells to an otherwise lethal environment. The adaptation to other osmotic stress as for example in the presence of sorbitol or glycerol appears to be mediated by the same mechanism, although this osmotic stress does not result in the killing of all cells and

allows growth to continue. Since all mutants showed growth on 2.5 M sorbitol supplement to YPD agar but not to 1.5 M NaCl supplement this suggests that all mutants examined (4.2.4.) are defective in the instantaneous osmoadaptation response. This was tested by diluting a middle log phase culture to 2 x 10^6 cells/mL in YPD + 1.5 M NaCl medium, and taking a sample for viable cell counts after 30 seconds. Except for the wild type, all *ssv* type alleles, namely *ssv1*-2, *ssv2*-1, *ssv3*-1, *ssv4*-1, *ssv5*-2, *ssv6*-1, *ssv7*-1, *ssv8*-2, *ssv9*-7, *ssv10*-1, *ssv11*-1, *ssv12*-1, *ssv13*-1, *ssv14*-1, *ssv15*-1, *ssv16*-1, *ssv*-100, *ssv*-123 and *ssv*-124 were not viable when plated out onto YPD agar plates. Also interesting is the fact that equiosmolar amounts of sorbitol have a differential growth inhibitory effect on the wild type.

4.2.6. Ca^{2+}-Sensitive Alleles Among *ssv* Mutants

Ca^{2+} uptake and homeostasis is facilitated by the vacuole, and a number of mutants deficient in this function have been isolated, most of which had vacuolar morphology or vacuolar ATPase defects (Kitamoto *et al.*, 1988; Ohya *et al.*, 1991). To test if the *ssv*-mutants exhibited similar defects, representative type alleles were streaked out onto freshly prepared YPD agar supplemented with 100 mM $CaCl_2$. Growth was recorded after 48 hours (Table 4.10). Only 3 alleles of the alleles tested exhibited a calcium sensitive phenotype (*cls*), of which the *ssv2*-1 mutant allele was only slowed in growth, but not completely defective. Growth of the mutant alleles *ssv3*-1 and *ssv4*-1 was inhibited completely by the concentration of calcium used. The three mutant alleles tested did have aberrant vacuolar morphologies, of which *ssv3*-1 did not have any vacuoles, and *ssv2*-1 had class B vacuoles. It is not known if the calcium sensitive growth defect was caused by a primary vacuolar defect. In the case of *ssv3*-1 it seems likely that the absence of a vacuolar compartment diminishes the cell's potential to regulate its cytoplasmic Ca^{2+} concentration. The partial growth defect of the vacuolar class B allele *ssv2*-1 might be a consequence of the disturbed assembly of the vacuolar compartment. Other definite class C alleles, e.g. *ssv9*-7, were not calcium sensitive. A possible explanation is that in this allele the homeostatic function of the vacuole is

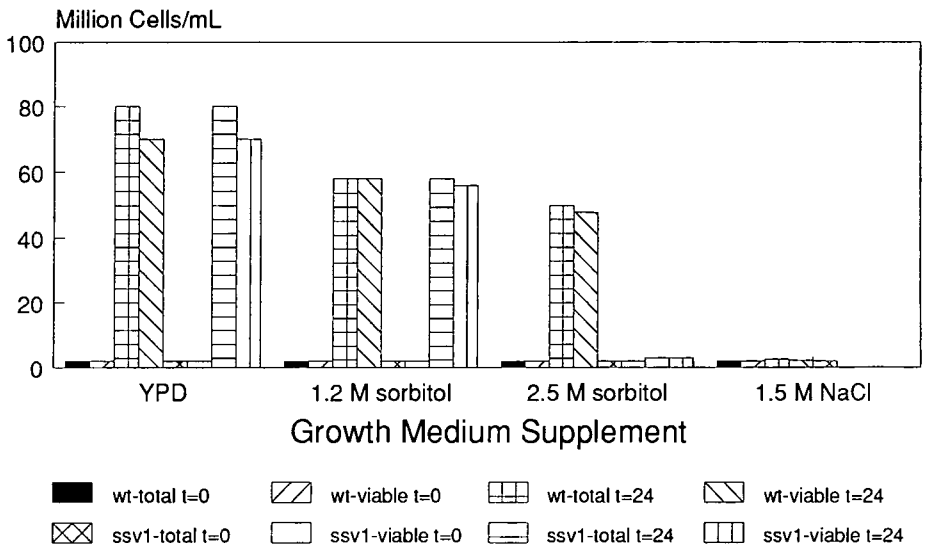
taken over by another compartment, or residual vacuolar or prevacuolar compartments function analogously. Previously isolated mutants defective in the vacuolar ATPase had a calcium sensitive phenotype and also were Pet$^-$ (Ohya *et al.*, 1991). Only one of the calcium sensitive *ssv* alleles, *ssv3*-1, is also defective for growth on non-fermentable carbon sources (4.2.7.). Considering its class C vacuolar phenotype it is unlikely its primary defect is in the vacuolar ATPase. The *ssv3*-1 allele is very slow in growth, and all other vacuolar sorting defects are the most severe observed, and it is likely that this mutant is a genuine vacuolar mislocalization mutant.

Type allele	Growth on YPD	Growth on YPD + 100 mM $CaCl_2$
wt	+	+
ssv1-8	+	+
ssv2-1	+	(+)
ssv3-1	+	-
ssv4-1	+	-
ssv5-2	+	+
ssv6-1	+	+
ssv7-1	+	+
ssv8-2	+	+
ssv9-7	+	+
ssv10-1	+	+
ssv11-1	+	+
ssv12-1	+	+
ssv13-1	+	+
ssv14-1	+	+
ssv15-1	+	+
ssv16-1	+	+
ssv-100	+	+
ssv-123	+	+
ssv-124	+	+

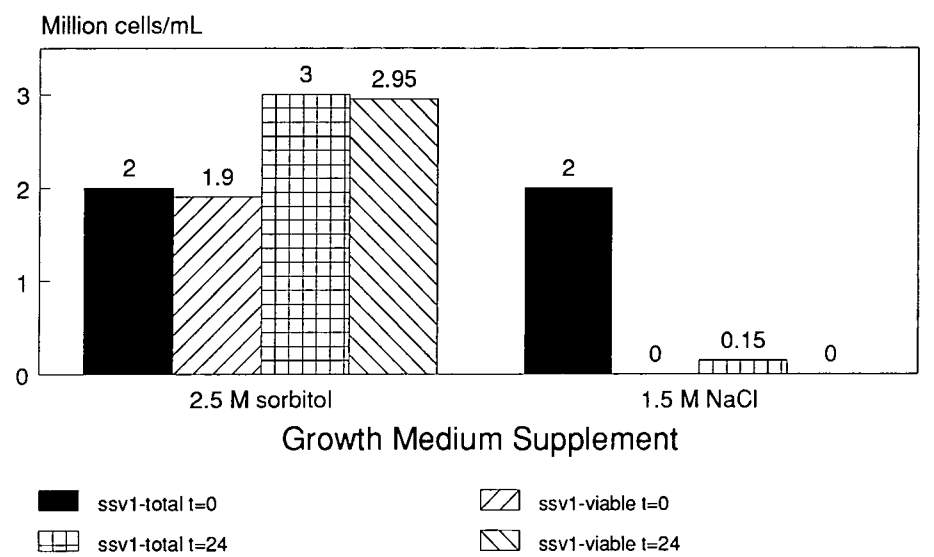
Table 4.10: *cls* phenotype of *ssv* mutants. Strains were judged for their ability to grow on YPD supplemented with $CaCl_2$ to 100 mM after 48 hours. Three alleles, *ssv2*-1, *ssv3*-1 and *ssv4*-1 were sensitive to 100 mM $CaCl_2$, where *ssv2*-1 displayed an intermediate phenotype in that it displayed partial growth under the above conditions. All other *ssv*-mutants displayed wild type growth behaviour.

Figure 4.5: Differential growth inhibition by equiosmolar concentrations of sorbitol and NaCl. Log phase SEY6210 (wt) and MLY0106 (*ssv1*-2) cells were diluted into YPD, YPD + 1.2 M sorbitol, YPD + 2.5 M sorbitol and YPD + 1.5 M NaCl. Growth was recorded immediately after dilution of the starter cultures and after 24 hours incubation at 30°C (A). To emphasize the lethal effect of NaCl on *ssv1*-2 the results described above are drawn to a different scale (B). Data of this analysis are the average from three independent measurements.

A


Million Cells/mL
100
80
60
40
20
0
YPD
1.2 M sorbitol
2.5 M sorbitol
1.5 M NaCl
Growth Medium Supplement
wt-total t=0
wt-viable t=0
wt-total t=24
wt-viable t=24
ssv1-total t=0
ssv1-viable t=0
ssv1-total t=24
ssv1-viable t=24

B


Million cells/mL
3
2
1
0
2
1.9
3
2.95
2
0
0.15
0
2.5 M sorbitol
1.5 M NaCl
Growth Medium Supplement
ssv1-total t=0
ssv1-viable t=0
ssv1-total t=24
ssv1-viable t=24

4.2.7. Growth on Non-fermentable Carbon Sources

A number of previously isolated *vps* mutants failed to grow on non-fermentable carbon sources such as glycerol (Raymond *et al.*, 1992) giving them an identical phenotype to mitochondrial Pet$^-$ mutants (Sherman, 1963). Since the vacuole might participate in some aspects of glycolysis (see Introduction), representative *ssv*-type alleles were tested for their Pet$^-$ phenotype on YPG medium (Table 4.11). A number of mutants, usually alleles with more severe *ssv* defects, also had a PET$^-$ phenotype. Except *ssv6*-1 and *ssv10*-1, which have a class A vacuolar morphology, all other alleles were either class B or class C. Some *vps* mutant alleles, usually with more severe morphological defects (Dulic and Riezman, 1989), were also defective for growth on glycerol. Their defect was not associated with a mitochondrial defect. All *ssv* mutants unable to grow on on glycerol as carbon source were not tested whether the defect was a result of a mitochondrial mutation.

Type allele	Growth on YPD	YPG
wt	+	+
ssv1-2	+	-
ssv2-1	+	+
ssv3-1	+	-
ssv4-1	+	+
ssv5-2	+	+
ssv6-1	+	-
ssv7-1	+	-
ssv8-2	+	+
ssv9-7	+	-
ssv10-1	+	-
ssv11-1	+	+
ssv12-1	+	+
ssv13-1	+	++
ssv14-1	+	+
ssv15-1	+	+
ssv16-1	+	+
ssv-100	+	-
ssv-123	+	-
ssv-124	+	-

Table 4.11: Growth of *ssv* mutant alleles on YPG. *ssv* type alleles were streaked out onto YPD (control) and YPG. Plates were incubated at 30°C for 48 hours. Generally Pet$^-$ mutants did not grow on the medium at all, unlike mitochondrial *petite* mutations which grow to small size colonies.

4.2.8. Temperature sensitive alleles

Since the osmosensitive phenotype and sensitivity to elevated temperature cosegregate in mutants lacking vacuoles (Banta *et al.*, 1988), and vacuolar function may also be required for growth at 37°C (Klionsky *et al.*, 1990), all osmosensitive mutants isolated in this study were tested for their ability to grow on YPD agar at putatively restrictive temperatures of 37°C and 39°C (Table 4.12).

Type allele	Growth after 48 hours at		
	24°C	37°C	39°C
wt	+	+	+
ssv1-8	+	+	+
ssv2-1	+	+	+
ssv3-1	+	-	-
ssv4-1	+	+	+
ssv5-2	+	+	+
ssv6-1	+	+	-
ssv7-1	+	+	-
ssv8-2	+	+	-
ssv9-1	+	-	-
ssv9-7	+	+	+
ssv10-1	+	+	-
ssv11-1	+	+	+
ssv12-1	+	+	+
ssv13-1	+	+	-
ssv14-1	+	+	+
ssv15-1	+	+	+
ssv16-1	+	+	-
ssv-100	+	+	+
ssv-123	+	+	+
ssv-124	+	+	-

Table 4.12: Temperature sensitive growth of *ssv* mutants. Type alleles of all *SSV*-complementation groups were streaked onto YPD agar and incubated at the indicated temperatures. The growth phenotype was scored after 48 hours incubation. Growth or temperature sensitive growth were unambiguous in that *ts* mutant alleles did not grow at all while temperature resistant alleles grew to large colonies.

It was found that most osmosensitive alleles did not confer temperature sensitivity to growth at 37°C. Only two complementation groups had a *ts* allele each which

cosegregated with the osmosensitive phenotype (*ssv3*-1 and *ssv9*-1), suggesting that the vacuole itself is not necessarily required for survival under elevated temperature stress. Also, light microscopic examination of *ssv3*-1 and *ssv9*-1 grown at both 24°C and 24°C which then were shifted to 37°C for 2 hours revealed that the mutants do not have vacuoles at either temperature. A number of mutants showed temperature sensitive growth at 39°C (Table 4.12). These mutants had generally severer vacuolar morphology defects than those that sustained growth at 39°C, although there is no direct correlation detectable between temperature sensitivity and vacuolar morphology. For example, *ssv9*-7, a vacuolar class C allele, did grow at 39°C. The cosegregation of the 39°C sensitive growth phenotype with the *ssv* phenotype was not assessed. The experimental findings dispute the previously suggested role of the vacuole in adaptation to elevated temperature (Banta *et al.*, 1988), since all severe class C alleles described in this study with one exception are not temperature sensitive. On the other hand, not all alleles of class C vacuolar mutants isolated so far are temperature sensitive, suggesting that temperature sensitive growth is a property of the aberrant gene product rather than a defect resulting from the absence of a vacuole.

4.2.9. Secretion of Vacuolar Proteinases

Mutants in each complementation group were tested for the secretion of one of several vacuolar proteinases in a non-lysing colony immunoblot. It was found that some alleles of each complementation group secreted several soluble vacuolar enzymes, namely CPY, proteinase A (PrA) and proteinase B (PrB), to different degrees (Table 4.13; Figure 4.6).

Table 4.13: Qualitative secretion of soluble vacuolar proteins.

Strain	Secretion of		
	PrA	PrB	CPY
SEY6210	-	-	-
SEY6211	ND	-	-
15113-8	ND	ND	++
14121-26	ND	ND	++
0552-11	++	+	++
MLY0101	+	ND	-

Table 4.13 - continued

Strain	Secretion of		
	PrA	PrB	CPY
MLY0103	+	-	+
MLY0104	+	-	+
MLY0107	ND	ND	+
MLY0108	ND	ND	+
MLY0111	++	++	++
MLY0116	++	++	++
MLY0117	+	+	+
MLY0120	++	++	++
MLY0121	+	+	-
MLY0124	-	+	+
MLY0126	ND	ND	+
MLY0134	+	-	++
MLY0139	+	+	+
MLY0202	++	++	++
MLY0304	++	++	++
MLY0307	ND	ND	++
MLY0309	++	-	++
MLY0310	++	+	++
MLY0402	ND	ND	+
MLY0416	+	+	-
MLY0419	+	+	++
MLY0508	++	++	++
MLY0512	-	-	-
MLY0513	(+)	-	-
MLY0522	+	ND	ND
MLY0524	ND	ND	+
MLY0526	++	++	+
MLY0527A	+	++	+
MLY0527B	+	+	+
MLY0601	ND	ND	+
MLY0701	++	++	++
MLY0702	-	-	-
MLY0704	ND	ND	++
MLY0705	(+)	-	-
MLY0709	(+)	-	-
MLY0712	+	+	++
MLY0713	+	+	-
MLY0716	+	ND	ND
MLY0725	-	-	+
MLY0729	++	++	++
MLY0731	-	+	+
MLY0732	+	+	+
MLY1001	ND	-	-
MLY1002	+	-	-
MLY1005	ND	-	-
MLY1006	+	-	-
MLY1105	+	+	ND
MLY1143	+	ND	ND
MLY1153	+	ND	ND
MLY1156	++	+	++
MLY1161	(+)	-	++
MLY1214	+	+	++
MLY1233	-	-	-
MLY1309	ND	ND	+
MLY1311	+	+	+

Strain	Secretion of		
	PrA	PrB	CPY
MLY1315	ND	ND	+
MLY1330	-	-	-
MLY1332	-	-	-
MLY1511	+	+	+

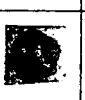
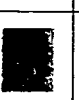
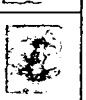
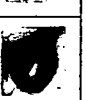
Table 4.13: Qualitative secretion of soluble vacuolar proteins in *ssv* mutants. Secretion of vacuolar proteinases was detected in a non-lysing colony hybridization blot. The secretory phenotype was assessed by comparison to the isogenic wild type strain SEY6210 as a negative control (-) and strain 0552-11 (*vps11*) as a positive control (+) which were included in every filter examined. ND = not determined.

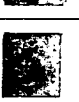
Although the colony blot allows detection of secretion of vacuolar proteinases, a quantification was hardly possible because different vacuolar precursors have different stability (Rothman and Stevens, 1986) and the different antisera used appeared to have different specific binding. The results of the immunoblot were quantified by immunoprecipitation of vacuolar proteinases from yeast sphaeroplasts and culture supernatant. It has been demonstrated before that sphaeroplasts behave identically to intact yeast cells in terms of protein secretion and protein sorting (Robinson *et al.*, 1988). This method has the advantage over using intact cells for pulse-chase experiments with subsequent sphaeroplasting in that accidentally lysed sphaeroplasts or cells do not distort the secretion pattern. The immunoprecipitations showed that vacuolar enzymes are mislocalized into the medium, although not necessarily to a high degree (Table 4.14). Alleles of complementation groups *ssv3*, *ssv9*, *ssv16* and *ssv-100* secreted more than 50% of CPY, *ssv1*, *ssv8*, *ssv10*, and *ssv15* mislocalized between 20 and 50% of CPY (Figure 4.7). All other complementation groups secreted CPY between 7 and 20%. The results show that only the 68 kDa CPY precursor is secreted, never the 61 kDa mature form. Therefore, the mislocalization occurs before vacuolar localization where the PrA mediated proteolytic processing of CPY takes place. Alleles of *ssv3* and *ssv9* (*ssv3*-1, *ssv9*-1 and *ssv9*-7) were unable to process intracellular CPY to its mature form, although PrA was present in both mutants. PrA (Figure 4.8) and PrB (Figure 4.9), the two other soluble vacuolar hydrolases tested for, are significantly secreted in only a

few mutants mutants. PrA is secreted in *ssv1*, *ssv12* and *ssv15* to any significat degree, although it accumulates as 48 kDa precursor in *ssv4*, *ssv5*, *ssv6*, *ssv7*, *ssv8*, *ssv9*, *ssv16* and *ssv*-100. Similarly, PrB is secreted only in *ssv1* and *ssv*-100 and accumulates as 42 kDa precursor in *ssv9* only (Table 4.14). The precursor form(s) of the respective proteinases are accumulated in some mutants (Figures 4.8 and 4.9). The *ssv3*-1 and *ssv12*-1 alleles examined for secretion of CPY by immunoprecipitation and autoradiography were unable to produce any significant amount of CPY during the pulse-chase period, although PrA and PrB were synthesized to a normal degree. Immunoprecipitated CPY from supernatant and intact spheroplasts was washed and SDS eluted as were the other strains, except that the radioactivity was determined by scintillation counting of both fractions. Pulse-chased vacuolar precursors of a wild type control retained over 95% of immunoprecipitable radioactivity intracellularly. The pattern of mislocalization in representative *ssv* mutants suggest that apart from *ssv1*, *ssv8*, *ssv9*, *ssv10*, *ssv16* and *ssv*-100 the other mutants do not mislocalize CPY to a significant degree. Among the significant secretors two are identical with known *vps* mutants (see above). PrA is secreted in *ssv1* and *ssv12* only, while PrB is secreted in *ssv1* and *ssv*-100 only. Thus only *ssv1* secretes all three vacuolar proteins tested for. PrA and PrB accumulate in a number of mutants suggesting that their mislocalization does not lead to secretion. Instead, PrA appears to be accumulated in a compartment different from the vacuole, while PrB appears to be matured in all mutants excepting *ssv9*. Since PrB maturation depends on PrA, this maturation must be catalyzed by residual vacuolar PrA (most mutants accumulating PrA have a small mature pool). Together these findings suggest the following: the *vps* defect in *ssv1* affects a compartment common to all three vacuolar enzymes. Other mutations secreting CPY except *ssv12* selectively affect the CPY sorting compartment (in *ssv*-100 also the PrB sorting compartment) and/or lead to an accumulation of PrA. This is indicative for differential sorting of all three enzymes, and also suggests that PrA and CPY are shuttled to the vacuole by a different mechanism. The *ssv* mutations in *ssv5*, *ssv8*, and *ssv*-100 might primarily affect the PrA trafficking machinery, resulting in some marginal effect on CPY sorting.

Figure 4.6: Non-lysing colony hybridization immunoblot of ssv mutants. Type alleles of each complementation group were tested for the secretion of PrA, PrB and CPY after blotting whole growing patches onto nitrocellulose over a period of 10 hours. The nitrocellulose blot was developed as described in Materials and Methods.

Colony blot assay testing for secreted vacuolar enzymes

Vac. Enzyme Secrtd.	wt	*ssv1*	*ssv2*	*ssv3*	*ssv4*	*ssv5*	*ssv6*	*ssv7*	*ssv8*	*ssv9*
PrA										
PrB					N.D.					
CPY					N.D.					

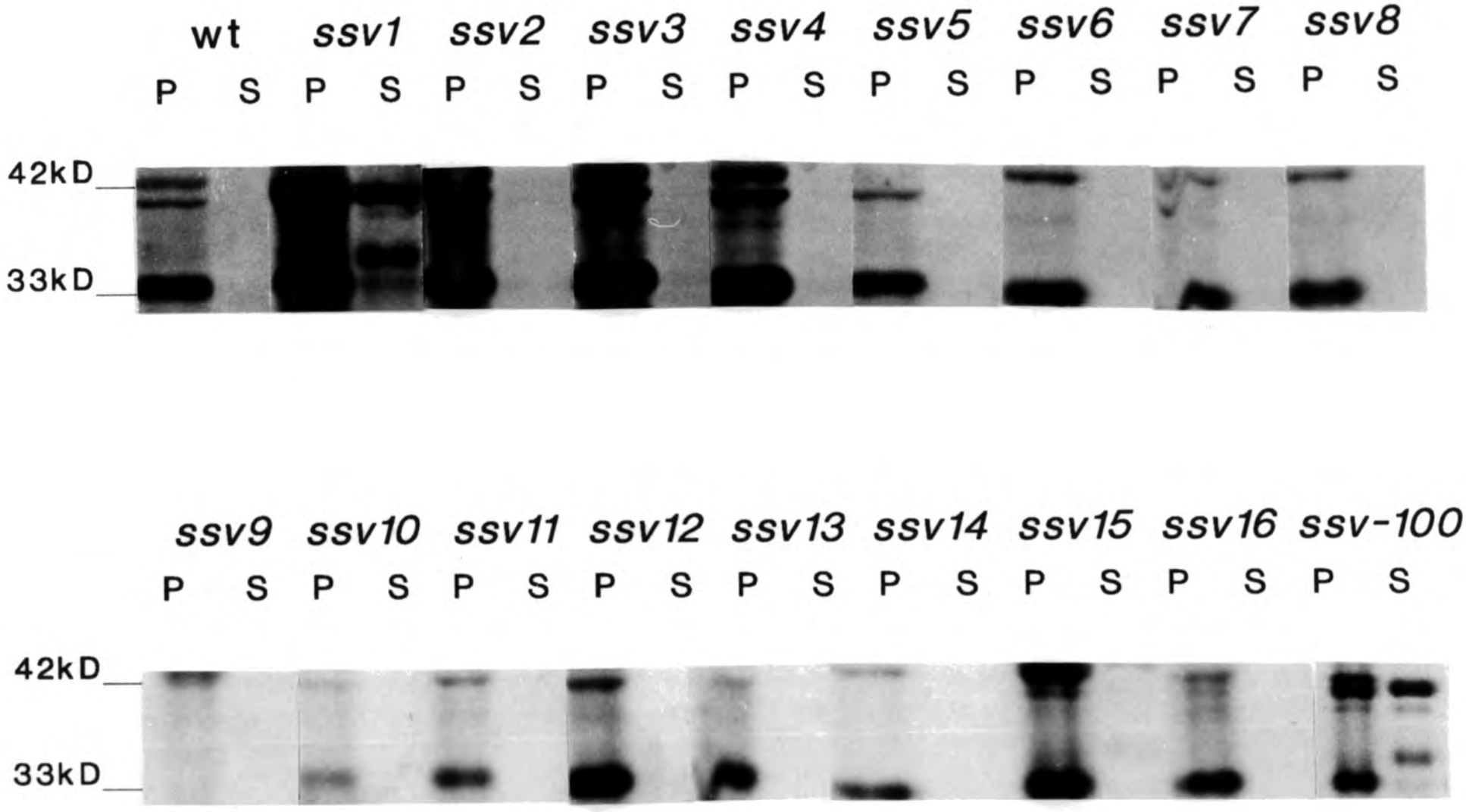
Vac. Enzyme Secrtd.	*ssv10*	*ssv11*	*ssv12*	*ssv13*	*ssv14*	*ssv15*	*ssv16*	*ssv-100*	*ssv-123*	*ssv-124*
PrA										
PrB						N.D.				
CPY						N.D.				

N.D. = Not Determined

Figure 4.8: Secretion of PrA in *ssv* mutants. Lanes P and S of each mutant show the immunoprecipitation of PrA from sphaeroplasts and culture supernatant respectively. Bands of approx. 48 kDa represent the PrA precursor, and at 42 kDa the proteolytically processed mature form is seen.


wt
ssv1
ssv2
ssv3
ssv4
ssv5
ssv6
ssv7
ssv8
48kD
42kD
P S P S P S P S P S P S P S P S P S
ssv9
ssv10
ssv11
ssv12
ssv13
ssv14
ssv15
ssv16
ssv-100
48kD
42kD
P S P S P S P S P S P S P S P S P S

Figure 4.9: Secretion of PrB in *ssv* mutants. Lanes P and S of each mutant show the immunoprecipitation of PrB from sphaeroplasts and culture supernatant respectively. Bands of approx. 42 kDa represent the CPY precursor, and at 33 kDa the proteolytically processed mature form is seen. The extra band around 40 kDa is probably an intermediate in PrB processing


wt
ssv1
ssv2
ssv3
ssv4
ssv5
ssv6
ssv7
ssv8
P S P S P S P S P S P S P S P S P S
42kD
33kD
ssv9
ssv10
ssv11
ssv12
ssv13
ssv14
ssv15
ssv16
ssv-100
P S P S P S P S P S P S P S P S P S
42kD
33kD

SSV	Relative secretion of α-gls	CPY	PrA	PrB	α-ms
WT	0%	0%	0%	0%	0%
ssv1-2	2%	51%	51%	32%	38%
ssv2-1	2%	10%	5%	3%	13%
ssv3-1[1]	2%	71%	3%	5%	42%
ssv4-1	5%	5%	5%	5%	46%
ssv5-2	3%	10%	4%	4%	25%
ssv6-1	1%	5%	1%	4%	17%
ssv7-1	4%	10%	1%	6%	21%
ssv8-2	3%	35%	8%	3%	102%
ssv9-7	1%	71%	8%	7%	47%
ssv10-1	7%	25%	2%	5%	44%
ssv11-1	1%	5%	5%	1%	54%
ssv12-1[1]	3%	33%	56%	5%	28%
ssv13-1	1%	4%	2%	4%	114%
ssv14-1	2%	7%	10%	6%	119%
ssv15-1	1%	22%	20%	10%	14%
ssv16-1	4%	80%	10%	1%	77%
ssv-100	5%	64%	5%	45%	75%
ssv-123	2%	ND	ND	ND	12%
ssv-124	1%	ND	ND	ND	41.6%

Table 4.14: Relative secretion of vacuolar hydrolases proteinase A, B, carboypeptidase Y, α-mannosidase and of α-glucosidase, a cytoplasmic marker. The relative secretion of α-mannosidase (α-ma) and α-glucosidase (α-gls) of periplasmic and cellular fractions of the *ssv* mutants was determined enzymatically, whereas the values for relative secretion of CPY, PrA and PrB were obtained by quantifying intracellular and secreted fractions on fluorographs using an LKB laser densitometer. ND = Not determined. [1]: % secretion was determined by immunoprecipitating vacuolar enzymes with subsequent scintillation counting (see text).

Mutant strains were transformed with the preproCPY-invertase fusion plasmid pCYI433 (Bankaitis *et al.*, 1986), encoding an in-frame fusion of prepro-CPY and invertase. This hybrid protein retains vacuolar targeting information. Transformants were subsequently assayed for the presence of the invertase activity in the total cell fraction and in the periplasm. The results show that in all *ssv* mutants some of the fusion peptide is mislocalized to the periplasm compared to the wild type where it is correctly delivered to the vacuole (Figure 4.10). Overall, the levels of secreted invertase are similar to those determined by immunoprecipitation of CPY, except in *ssv3* and *ssv13* where significantly less of the CPY-Inv fusion is mislocalized than CPY, and in *ssv4*

and *ssv7*, where the mislocalization effect of the fusion peptide is more severe than that of CPY. All heterozygous diploids of representatives of each complementation group, which harboured pCYI433, were recessive for the mislocalization of the CPY-Inv fusion (Figure 4.10). However, some representative alleles were not fully complemented by the wild-type allele. *ssv10*, *ssv11*, *ssv12* and *ssv15* were fully complemented whereas *ssv16*-1 still showed 20% secretion of CPY-Invertase in the heterozygous diploid. These particular mutants are truly recessive for the osmosensitive phenotype (Table 4.3 and 4.4).

Mutants were also tested for mislocalization of α-mannosidase into the periplasm or the cell surface (Table 4.14). Although recent evidence suggests that this membrane associated vacuolar enzyme does not transit through the secretory pathway to the vacuole (Yoshihisa *et al.*, 1990), all *ssv* mutants mislocalize α-mannosidase at significantly higher levels than the wild-type. *ssv8*, *ssv13* and *ssv14* mislocalize 100% of the total α-mannosidase activity. In the other complementation groups the levels of mislocalization are similar to that of the soluble vacuolar enzymes.

All mutants retained α-glucosidase, a cytoplasmic marker enzyme, strongly suggesting that the vacuolar protein sorting defect is genuine and not due to leakage of cytoplasmic content (Table 4.14). This result is supported by the fact that all *ssv* mutants examined for secretion of vacuolar proteinases only secrete the p2-forms of vacuolar proteins but not the unglycosylated pp-forms, suggesting that the presence of the vacuolar proteinases in the external fraction is due to a missorting defect at a post-Golgi stage rather than a leakage of freshly synthesized precursor.

4.2.10. Cosegregation of Mutant Phenotypes

Random spores isolates of each back-crossed mutant strain were examined for cosegregation of the different mutant phenotypes. In only two cases (*ssv3*-1 and *ssv9*-1), temperature sensitivity, vacuolar morphology, osmosensitivity and secretion of one or more vacuolar enzymes cosegregated and probably are allelic. A cosegregation of osmosensitivity, vacuolar morphology and the secretion of vacuolar hydrolases in all

other non-temperature sensitive alleles of all complementation groups where examined could be demonstrated (Table 4.15).

Strain	*OSM*	*osm*	*vps*	*ts*	*vam*
MLY0101		38	38	0	38 A
	42		0	0	42 A
MLY0103		13	13	0	13 A
(*ssv10*-1)	67			0	67 A
MLY0111		13	13	0	ND
(*ssv2*-2)	35		0	0	ND
MLY0117		7	7	0	ND
(*ssv2*-1)	1		0	0	ND
MLY0121		6	6	0	ND
	10		0	0	ND
MLY0124		0	0	0	ND
(*ssv11*-1)	24		ND	0	ND
MLY0134		11	11	0	ND
(*ssv8*-1)	13		0	0	ND
MLY0139		9	9	0	9 A
(*ssv12*-1)	71		0	0	71 A
MLY0304		5	5	0	ND
(*ssv1*-6)	11		0	0	ND
MLY0309A		16	16	0	ND
(*ssv7*-1)	32		0	0	ND
MLY0310		5	5	5	$5A^r$
(*ssv9*-1)	19		0	0	ND
MLY0416		10	5	0	ND
	14		0	0	ND
MLY0512		34	34	0	34 A
	46		0	0	46 A
MLY0513		5	0	0	ND
(*osm3*-1)	24		0	0	ND
MLY0522		6	6	0	ND
	2		0	0	ND

Strain	*OSM*	*osm*	*vps*	*ts*	*vam*
MLY0526		5	5	0	5 A
(*ssv13*-1)	75		0	0	75 A
MLY0527A	24	24	24	ND	ND
(*ssv6*-1)	0	ND	ND	ND	ND
MLY0527B		12	12	12	ND
	9		0	0	ND
MLY0701		30	30	0	30 A
(*ssv14*-1)	60		0	0	30 A
MLY0705		11	0	0	ND
(*osm4*-1)	12		0	0	ND
MLY0709		5	0	0	ND
(*ssv19*-1)	3		0	0	ND
MLY0713		6	6	0	ND
	9		0	0	ND
MLY0716		4	4	0	ND
	20		0	0	ND
MLY0729		12	12	0	ND
(*ssv5*-2)	12		0	0	ND
MLY0731		0	ND	ND	ND
(*ssv5*-3)	16		ND	0	ND
MLY0732		11	11	11	ND
(*ssv3*-1)	13		0	0	ND
MLY1002		3	3	0	ND
	21		0	0	ND
MLY1105		15	15	0	ND
(*ssv7*-2)	9		0	0	ND
MLY1143		5	5	0	ND
(*ssv1*-8)	3		0	0	ND
MLY1153		2	2	0	ND
(*ssv1*-9)	22		0	0	ND
MLY1156		0	ND	ND	ND
(*ssv16*-1)	8	0	0	0	ND

Strain	*OSM*	*osm*	*vps*	*ts*	*vam*
MLY1161		7	0	0	ND
(*ssv20*-1)	17		0	0	ND
MLY1214		3	3	0	ND
(*ssv9*-6)	5		0	0	ND
MLY1233		18	18	0	ND
	6		0	0	ND
MLY1311		6	6	0	ND
(*ssv9*-7)	10		0	0	ND

Table 4.15: Cosegregation of pleiotropic phenotypes in *ssv*-mutants. Random spore isolates were tested for osmosenstive growth (*osm*), vacuolar protein sorting defects (*vps*), temperature sensitive growth (*ts*) and aberrant vacuolar morphology (*vam*). The *vps* phenotype was determined in a non-lysing colony dot-blot, the vacuolar morphology was assessed under Normarski-optics.

Figure 4.10: Cosegregation of osmosensitive and vacuolar protein sorting defective phenotype in *ssv* mutants. Random spore isolates of heterozygous diploids between *ssv* mutants and their isogenic parent strain were tested for their phenotypes by subjecting each isolate to a series of tests (see text). The photo depicts a non-lysing colony dot-blot on several random spore isolates. The isolates were also tested for their sensitivity to 1.5 M NaCl and their growth at elevated temperature. Phenotypic traits were said to cosegregate if all spore isolates were either wild type for all phenotypic characters or all had all mutant phenotypes.

4.2.11. Dominance/Recessiveness of Phenotypes

All pleiotropic phenotypes of the *ssv* mutants isolated and characterized so far are recessive with one exception: the *vps* phenotype of the *ssv16*-1 allele is semi-dominant (Figure 4.11). Especially no dominant osmosensitive phenotype was observable as is the case for a *act1*-1 mutant allele (Tables 4.3 and 4.4). The dominance/recessiveness of the vacuolar defect in heterozygous diploids was not examined by subjecting the strains to a detailed electron microscopic examination. However, under Normarski interference microscopy all heterozygous diploids of *ssv* mutants and their isogenic wild type appeared to have vacuoles. Other studies (Banta *et al.*, 1988) also suggest that vacuolar morphology defects are recessive in nature.

4.2.12. Comparison With Other Osmosensitive Alleles

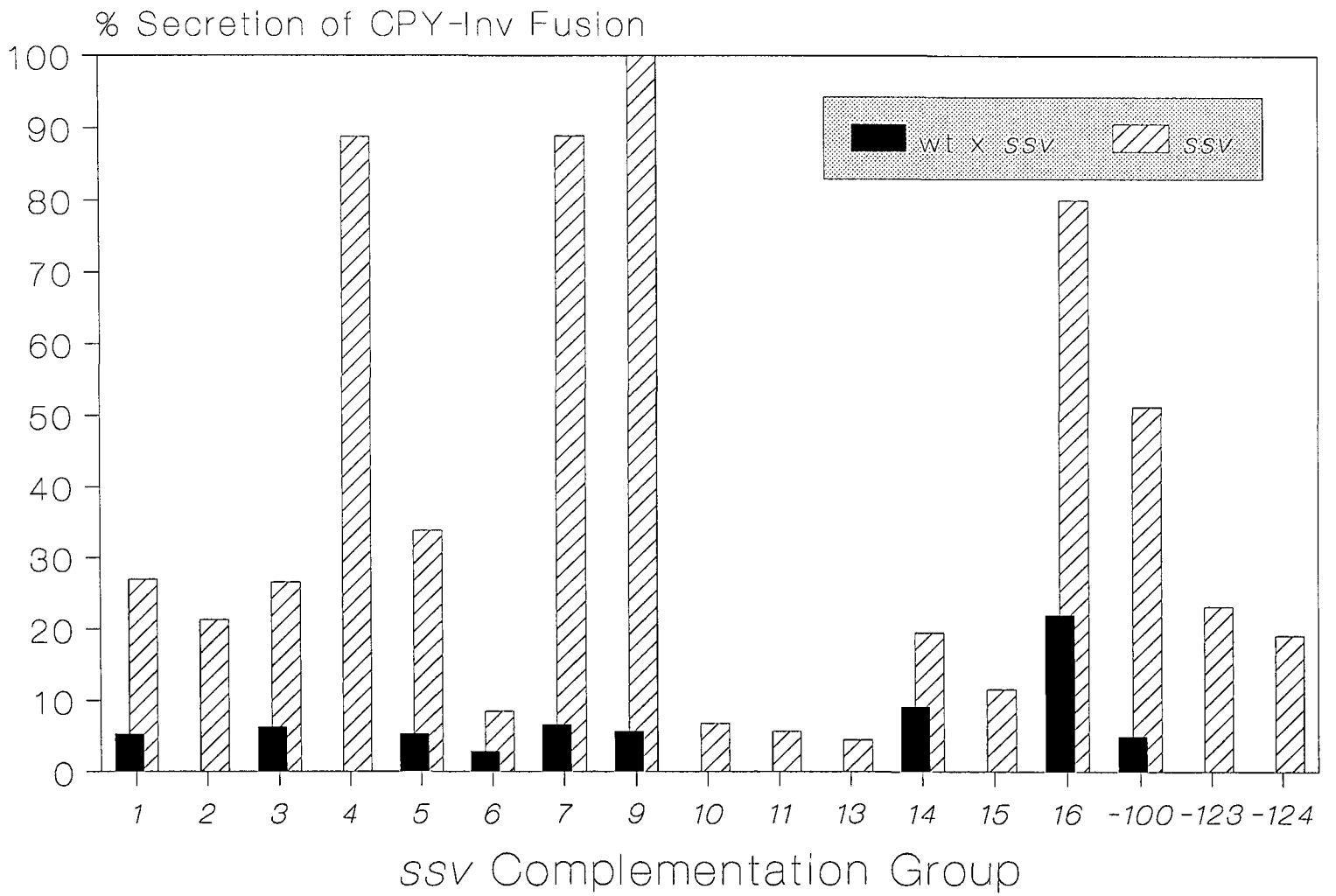
A number of mutants with defective vacuolar function other than the ones described here have been isolated, such as vacuole inheritance (Weisman *et al.*, 1990), vacuolar morphology (Wada *et al.*, 1988), amino acid storage (Kitamoto *et al.*, 1988), ion transport (Ohya *et al.*, 1986), and acidification of the vacuole (Preston *et al.*, 1989; Yamashiro *et al.*, 1990). A partial genetic overlap of mutants deficient in different vacuolar functions with mutants defective in correct vacuolar protein targeting has been observed, stressing the complexity of vacuolar functions (Klionsky *et al.*, 1990). Osmosensitivity has been reported in a number of different mutants, as for example *act1*-1 (Novick and Botstein, 1985), *osm1*, *osm2* (Singh and Sherman, 1978) and some *vps* mutants (Klionsky *et al.*, 1990). All alleles of each *ssv* complementation group were tested for complementation of the osmosensitive phenotype against all available previously identified osmosensitive mutants, namely *vps1*, *vps11*, *vps15*, *vps16*, *vps18*, *vps33*, *vps34*, *act1*-1, and *osm2* (Tables 4.3 and 4.4). Except *ssv1*, which is allelic to *vps1*, and *ssv15*, which is identical to *osm2*, none of the other previously identified osmosensitive alleles overlap with the ones isolated in this study based on complementation of the associated osmosensitive growth phenotype. Representative *ssv*

mutants of each complementation group were tested for complementation of the Vps phenotype against representatives of all 47 *vps* complementation groups (Rothman *et al.*, 1989) using a non-lysing colony dot-blot of purified diploids (M. Latterich and M.D. Watson, unpublished results; I. Howald-Stevenson and T.H. Stevens, unpublished results). Except *ssv1*-2, which is allelic to *vps1*, *ssv9*-7 which is identical to *vps33* none of the other *ssv* alleles are identical to any of the remaining *vps* genes (Table 4.16). It appears that the *ssv1*-7 allele is identical to *vps33*, also judged by its non-complementation of the *vps* phenotype (I. Howald-Stevenson and T. Stevens, personal communication). The allelism of the three mutant alleles could for the before mentioned reason not be confirmed. Complementation analysis of the *ssv* collection against the *vps* mutants screening for complementation of the *vps* phenotype confirmed that with the afore mentioned exceptions the *ssv* mutants are not identical to all known *vps* mutants (I. Howald-Stevenson and T. Stevens, personal communication).

Also, none of the *ssv* mutants appears to be allelic to the *act1*-1 gene, since the *ts* phenotype is complemented by all *ssv* mutants (the osmosensitive phenotype is dominant in *act1*-1). Mutants in three complementation groups defective in plasma membrane ATPase function are also osmosensitive (McCusker *et al.*, 1987). Although no vacuolar defects have been reported in these mutants there remains the possibility that some of the *ssv* mutants are defective in a similar function and their vacuolar defects are of a secondary nature.

Other vacuolar mutants, of which none were sensitive to osmotic stress, were selectively sensitive to cations such as Ca^{2+} at elevated concentrations (Ohya *et al.*, 1986). Although Ca^{2+} sensitivity has been associated with some vacuolar morphology mutants before (Wada *et al.*, 1988), only three of the *ssv* alleles are moderately sensitive to the divalent cation (*ssv2*, *ssv3*, and *ssv4*) of which only *ssv3* lacks a vacuole (Table 4.10). Other vacuolar mutants were not available for complementation tests, and any statement/comparison is not possible at this stage.

Figure 4.11: Complementation of vacuolar protein mislocalization in ssv mutants in heterozygous diploids, using the CPY-Invertase fusion plasmid to quantify the vacuolar mislocalization defect. The values of relative secretion were obtained by division of the periplasmic fraction by the total invertase activity and corrected against the residual wild type activity. *ssv*-123 and *ssv*-124 (sterile) were not tested for complementation of the vacuolar protein mislocalization effect since they could not be crossed against the wild type. Each value represent the average of two independent estimations.


% Secretion of CPY-Inv Fusion
100
90
80
70
60
50
40
30
20
10
0
wt x ssv
ssv
1
2
3
4
5
6
7
9
10
11
13
14
15
16
-100
-123
-124
ssv Complementation Group

ssv Mutant Allele	Genetic Overlap
ssv1-2	*vps1*
ssv1-8	*vps1*
ssv1-7	*vps33*
ssv9-2 to -7[1]	*vps33*
ssv15-1	*aro7-osm2*

Table 4.16: Genetic overlap between *ssv* mutants with previously isolated mutants. [1]: The *ssv9*-1 allele complements the *vps33* mutant and is thus not allelic.

4.2.13. Other Associated Phenotypes

There are other phenotypes associated with some mutants isolated in this study. So far most strains have only been analysed for vacuole biogenesis associated defects, but during routine procedures other pleiotropic defects were found. Two mutant strains, MLY0202 (*ssv*-124) and MLY0712 (*ssv*-125), were sterile judged by their inability to form diploids with any strain of opposite mating type (Table 4.3).

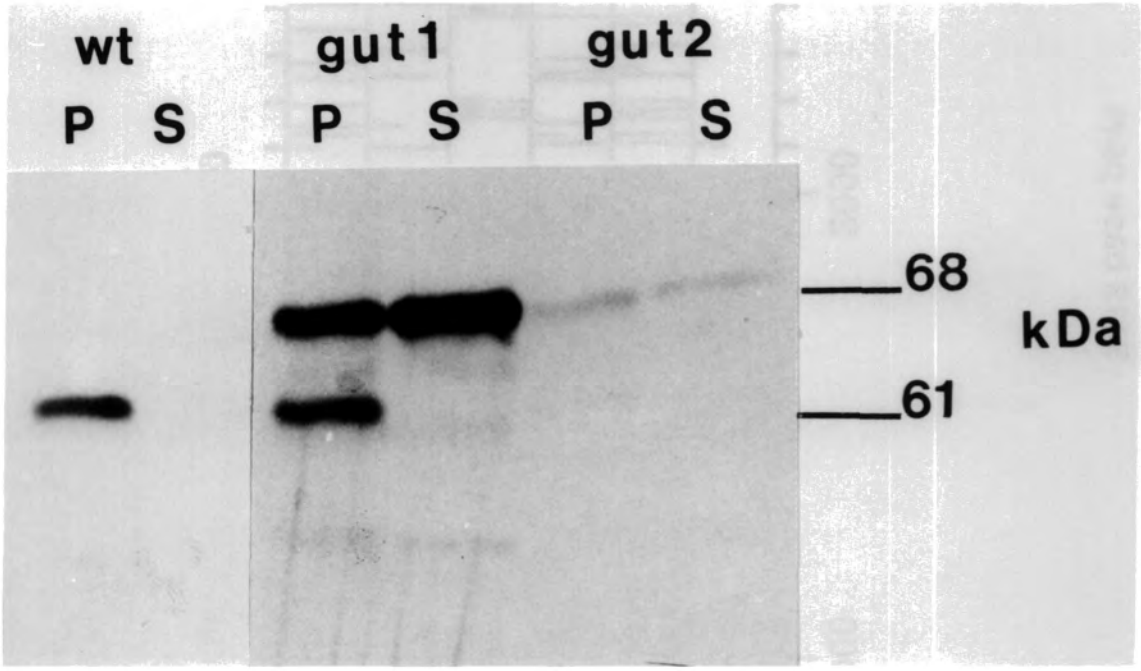
Most *ade2* strains lacking intact vacuoles did not accumulate the pink *ade2* fluorophore giving the strains an *ADE2* appearance. This observation has been exploited to isolate some of the *vam* mutants (Wada *et al.*, 1988). The *ade2 ssv7*-1 leads to an excessive accumulation of the *ade2* fluorophore giving the strain a reddish appearance. The strain also has other associated defects, such as aberrant nuclear morphology and a cell cycle defect. The gene corresponding to the mutation has been cloned and sequenced (Chapter 6).

4.2.14. Mutants in Metabolic Pathways With *ssv*-like Phenotypes

The fact that *osm2-aro7*, a mutant defective in chorismate mutase activity, is allelic to a *ssv* mutant is intriguing, and the *ssv* phenotype of the mutant has been examined in more detail (Chapter 7). A primary defect in chorismate mutase, an enzyme involved in aromatic amino acid biogenesis, results in a number of secondary

vacuole associated defects such as vacuole morphology and the secretion of vacuolar enzymes. This represents evidence for the vacuolar biogenesis being subject to metabolic control. One characteristic of previously isolated *vps* mutants is their defect in gluconeogenesis, and the question arises whether their primary defct is in vacuolar protein sorting or in gluconeogenesis. Also, the adaptive osmoregulatory response in *S. cerevisiae* is mediated by glycerol accumulation (Blomberg and Adler, 1989). Two mutants with defects in glycerol catabolism, *gut1* and *gut2*, have been isolated and found to be defective in glycerol kinase and glycerol phosphate dehydrogenase activity, respectively (Sprague and Cronan, 1977). The mutants were examined for their vacuole associated phenotypes to examine whether mutations in *GUT1* and *GUT2* were osmosensitive for growth or had vacuolar defects as a secondary response. While both mutant alleles had normal vacuoles judged by immunofluorescence (data not shown), they were moderately osmosensitive for growth on YPD supplemented with 1.5 M NaCl compared to the wild type, but not as much as the other *ssv* mutants isolated in this study (data not shown). Both *gut1*-1 and *gut2*-1 had a strong *vps*-phenotype in that they secreted up to 40% p2CPY and were unable to mature a large proportion to the vacuolar form (Figure 4.12). The question arises whether two different *vps* defects cause the *gut1* and *gut2* phenotype, or whether defects in glycerol catabolism provoke a *vps* phenotype. The nature of the primary defect in the two mutants makes it appear unlikely that the latter is true.

Figure 4.12: Secretion of CPY in SEY6210 (wild type), S5 (*gut1*) and S22 (*gut2*). Lanes P and S of each strain show the immunoprecipitation of CPY from sphaeroplasts and culture supernatant respectively. Compared to the wild type which has only 61 kDa matured CPY present intracellularly, both gut1 and gut2 secrete a large proportion of the 68 kDa precursor form of CPY (S) and also fail to mature part of their intracellular CPY (P).


wt
gut 1
gut 2
P S
P S
P S
68
61
kDa

4.3. Discussion

A number of osmosensitive mutants in *S. cerevisiae* were isolated in an effort to identify and isolate genes involved in vacuole biogenesis and osmoregulation. Standard complementation analysis could assign the mutants to at least 23 complementation groups. A significant number of mutants showed weak complementation to alleles of most of the 22 groups and thus appear to have a semi-dominant phenotype. As most of these mutations fail to complement each other, they may all be members of a complementation group, *ssv17*. Their novel complementation properties make this group a potentially interesting one for further investigation. Although the genetic tests employed in this study deserve caution when interpreting their results, it was observed that a large number of mutants did not complement mutant alleles of several distinct complementation groups. This non-complementation was allele specific, and is characteristic for proteins forming a multimeric complex. Two sterile osmosensitive mutants were also isolated (*ssv*-123 and *ssv*-124), but they have not been characterized further. Subsequent electron microscopic analysis of all mutants showed that alleles of 17 complementation groups had an altered vacuolar morphology similar to that observed by Banta *et al.*, 1988, with five groups having alleles completely lacking a vacuole. A number of aberrant subcellular structures were detectable in some mutant strains such as autophagic vacuoles, aberrant membraneous structures with similarity to Berkeley bodies representing accentuated Golgi structures, and small vacuole-like compartments. These are similar to "organelles" that also accumulate in *end2* mutants which have been suggested to represent an intermediate in endocytosis (Chvatchko *et al.*, 1986). The nature of this compartment has not yet been examined, although it should be fruitful to attempt an isolation and characterization. Its appearance using the reduced osmium-thiocarbohydrazide-reduced osmium procedure differs from that of vacuoles in that it has a center of low electron density, unlike vacuoles which have an uniform electron-dense appearence except with a confined region of electron transparancy representing polyphosphate crystals. Some mutant alleles contained a large

number of these compartments giving them the appearance of class B mutants. It would be tempting to speculate that these compartments represent a pre-vacuolar endosome. Some vacuolar class B mutants do not possess this compartment. In these mutant strains the putative prevacuolar compartment cauld fail to fuse with the vacuole, thus class B vacuoles representing "mature" prevacuolar compartments. The fusion of these compartments with the vacuole has been observed in some mutants, though not yet in wild type cells. If this fusion process occurs as part of normal vacuole biogenesis the fusion might be delayed in these mutants allowing the intermediates to be detected by electron microscopy. A less likely alternative explanation is that these compartments represent peroxysomes because the fusion of peroxysomes with the vacuole has been reported before (H.-L. Chiang, personal communication). However, peroxisomes should not be abundant or present at all in yeast grown on high glucose concentrations. A 135 kDa glycoprotein (vgp135) has been located to a probably prevacuolar compartment (Nishikawa *et al.*, 1990). Immunolocalization of this protein to the putative prevacuolar compartment described here could test this hypothesis.

Although a new vacuolar morphology classification scheme has been presented elsewhere taking account of the vacuolar membrane ATPase assembly based on immunofluorescence methods (Raymond *et al.*, 1992), the morphological criteria in this study are largely based on electron microscopic data, partly because an assessment of other subcellular features was thought to be important and to be sufficient to adhere to the one proposed by Banta *et al.*, 1988. The knowledge of detailed phenotypes, such as different vacuolar morphology, permits the application of epistasis analysis (Botstein and Maurer, 1982). However epistasis analysis is only feasible if the mutations to be analysed affect a linear pathway, such as genes in the secretory pathway. Vacuolar morphology could be subject to a number of independent pathways or bypass pathways and thus complicating the othewise informative analysis. Also, epistasis analysis requires the construction of double mutants by genetic means involving tetrad dissection, which was not possible to carry out during the time of this study.

Some alleles of the identified complementation groups secreted several soluble vacuolar hydrolases and also α-mannosidase, a membrane associated vacuolar enzyme,

was mislocalized to the cell surface. The vacuolar protein missorting defect in all but 4 cases (*ssv1*-2, *ssv8*-1, *ssv9*-7 and *ssv*-100) was not as severe as in most other *vps* mutants previously isolated (Robinson *et al.*, 1988; Rothman *et al.*, 1989). Excepting one complementation group (*ssv15*), mutants of a class C phenotype had more severe vacuolar protein mislocalization defects than alleles with a class A or class B phenotype. *ssv16*-1 (class B) secretes CPY and α-mannosidase at a comparable level to class C alleles. Mutants of vacuolar morphology class A and B still have an intracellular pool of mature CPY but secrete the precursor of CPY. Since the PrA dependent processing of CPY occurs in the vacuole, these mutants still have biochemically intact vacuoles, or functionally similar compartments. Thus the alleles which secrete only a fraction of the total vacuolar hydrolases, may have mutations that cause inefficient delivery of vesicles destined for the vacuole or change the fidelity of the sorting process as a result of another mutation in a different vacuolar function. Alleles of two complementation groups (*ssv3* and *ssv9*), which have no vacuoles, are unable to process the CPY precursor to the mature form and secrete a large proportion of it. This is most easily explained by the lack of mature PrA found in these strains. In this context it appears to be remarkable that *ssv15* lacks any detectable vacuole as judged by electron microscopy and still is able to mature a large proportion of the CPY synthesized (see Chapter 7). All other *ssv* mutants examined for their mislocalization and intracellular accumulation of PrA, PrB and CPY were able to mature all of the intracellularly localized vacuolar proteins, and only traces of vacuolar proteinase precursors were localized to the cell surface. Only *ssv1*-2, *ssv16*-1, *ssv*-100, *gut1* and to some extent *gut2* accumulated both precursor and mature proteinases intracellulaly. Most vacuolar class B *ssv* alleles examined for their quantitative secretion of vacuolar proteinases secrete very little precursor, unlike previously isolated class B *vps* mutants which secrete vacuolar proteins almost quantitatively (Robinson *et al.*, 1988). This difference is probably due to the mutant isolation strategies developed. Vacuolar class B alleles identified previously were isolated on the basis of a vacuolar protein missorting defect (Bankaitis *et al.*, 1986; Banta *et al.*, 1988). It is likely that the sorting defect results in an aberrant vacuolar morphology because putative proteins needed for vacuolar integrity of

fusion are not localized correctly. Class B *ssv* mutants were isolated on the basis of an osmoregulatory defect. While *vps* mutants are probably defective in sorting at the Golgi level or the putative receptor recycling and thus secrete vacuolar proteins, the *ssv* mutants isolated in this study could be defective in any hypothetical vacuolar function. While sorting in these mutants still can occur, subsequent requirements for vesicle transport and fusion might be disturbed in the mutation. Assuming unidirectionality of vesicular transport from Golgi to the vacuole and that it represents the main route by which the vacuole derives its constituents, the mutants will fail to form vacuoles but still may be able to perform part of their function in a prevacuolar compartment. Though no evidence for this hypothesis can be provided here, the accumulation of vacuole like compartments in some mutant alleles that failed to assemble a vacuole could represent such intermediates in vacuole biogenesis.

Members of complementation groups *ssv3* and *ssv9* mislocalize not more than fifty percent of α-mannosidase although they lack a structurally recognizable vacuole. Mutants in other complementation groups, *ssv8* (class B), *ssv13* (class B) and *ssv14* (class A), mislocalize up to 100% of α-mannosidase, whereas less than 40% of soluble vacuolar enzymes are secreted. These findings are similar to previous studies of *vps* mutants which showed that only up to 50% of the total vacuolar hydrolase is secreted in four class C complementation groups (Robinson *et al.*, 1988). The intracellular location of α-mannosidase and the soluble vacuolar enzymes was not further investigated. α-mannosidase is not delivered to the vacuole via the secretory pathway, but it might be able to insert into the vacuole directly (Yoshihisa and Anraku, 1990). The simplest explanation for the mislocalization of α-mannosidase is that a hypothetical vacuolar transmembrane protein associated with the import of α-mannosidase is mislocalized to the cell surface causing α-mannosidase to subsequently insert into the plasma membrane. This explanation raises the question of how a vacuolar membrane protein can be sorted to the cell surface. Among several possibilities three appear plausible: vacuolar membrane proteins are actively sorted to the vacuole, and a mutation affecting part of the sorting process will result in a secretion of the proteins through the default pathway. Since membrane bound and even different soluble vacuolar proteins are sorted

independently (Klionsky *et al.*, 1990; Paravicini *et al.*, 1992) this interpretation of the results obtained in this study seems plausible. The mislocalization of other vacuolar membrane proteins has not been assessed. The active sorting process of membrane associated vacuolar proteins has recently been questioned (Raymond *et al.*, 1992). Secondly, a cytoplasmic protein could mediate import of α-mannosidase by associating with the vacuolar membrane and directing the protein into the vacuole without having itself to take the secretory pathway. In *ssv8*, *ssv13* and *ssv14* this protein could associate with the plasma membrane and direct translocation of α-mannosidase through the membrane. This model is supported by the fact that in early *sec* mutants translocation of α-mannosidase still occurs efficiently and there seems not to be a requirement for the import machinery to be translocated through the secretory pathway although they could be present on the vacuolar membrane before the blocking the secretory pathway is imposed (Yoshihisa and Anraku, 1990). Thirdly, the substrate for the α-mannosidase assay, p-nitrophenyl-α-D-mannopyranoside, could be transported into intact cells and hydrolysed in the cytoplasm or in the vacuole more efficiently than in the wild type. This explanation does not account for the fact that α-glucosidase, a cytoplasmic marker in yeast, is retained in the cell, arguing against a leaky plasma membrane. Several mutants such as *vac1* to *vac4* (Shaw and Wickner, 1991; Weisman *et al.*, 1990), *vps3* (Raymond *et al.*, 1990) and *vps34* (Herman and Emr, 1990) have been identified which appear to have delayed segregation of vacuoles to the growing bud. A few *ssv* isolates, as judged by light and electron microscopy, also show this defect. With the exception of *vac2*, which has a segregation defect but not a sorting defect, the other segregation mutants have also a *vps* phenotype. One possible explanation that is consistent with all available experimental data is that the sorting defect is responsible for the mislocalization of a component that serves the attachment onto the segregation apparatus to the cell surface. As an immediate result the vacuole is unable to segregate, and at a later stage will form *de novo* from Golgi derived material. The distribution of organelles seems to be mediated either by cytoskeletal elements, cytoplasmic movement or by attachment to the plasma membrane (Yaffe, 1991). Assuming that one of the cytoskeletal features attached to the vacuole is mutated in *ssv* mutants exhibiting a

segregation defect but not a sorting defect the osmosensitive phenotype can be explained by that either the vacuole will be destabilized under low water stress or that cytoskeletal mutants, like actin, are intrinsically osmosensitive irrespective of osmoregulatory function. There is evidence that both vacuoles and the mammalian lysosomes are associated with cytoskeletal proteins. Dynein has been localized to lysosomes (Lin and Collins, 1992) as has actin (Mehrabian *et al.*, 1984) and microtubules (Matteoni and Kreis, 1987), of which the attachment of the latter is mediated by a 50 kDa protein in an ATP dependent process (Mithieux and Rousset, 1989). Also, there is evidence that the organelle shape is dependent on movement along microtubules (Kachar *et al.*, 1987). Thus, cytoskeletal elements are known to be associated with lysosomes and also to mediate movement of cytoskeletal elements. The unambiguous association of microtubules with vacuoles has not yet been demonstrated, and thus the formal possibility remains that vacuolar morphology as well as vacuolar segregation depends on some cytoskeletal element, possibly different from microtubules.

By selecting for mutants involved in osmoregulation, mutants deficient almost exclusively in several vacuolar functions were isolated. This seems to suggest that in most cases osmohomeostasis and other vacuolar functions, such as protein sorting, vacuole biogenesis, or correct vacuolar assembly are inextricably linked, or that a large number of genes participate in vacuole mediated osmoregulation. Whether the osmoregulatory defect is primary and results in a number of secondary defects such as aberrant vacuole biogenesis or is a secondary defect as a consequence of vacuolar defects is difficult to judge at present, but evidence suggest that the latter is more likely. For example the absence of the vacuole (class C) always confers an osmosensitive phenotype as a result (Banta *et al.*, 1988). However, three *vps* complementation groups and at least 18 *ssv* groups, which do not have a class C phenotype, also have osmosensitive alleles. This suggests that in cases where a vacuolar structure exists, only certain functions within these vacuoles are required for osmoregulation. Any defect in delivery of these osmotically active components to the vacuole, rather than vacuole biogenesis *in toto*, will cause an osmosensitive phenotype. Alternatively one gene

product could be required for different independent functions such as osmohomeostasis and vacuole biogenesis. The concept of bi- or multifunctional proteins is not new, and a recent study has assigned several roles to the endoplasmic reticulum protein disulphide isomerase (PDI) catalysing disulphide bridge formation and also representing the glycosylation site binding protein (LaMantia *et al.*, 1991; Noiva and Lennarz, 1992). The PDI/GBP protein represents one protein with at least two separate, independent functional domains one of which is a catalytic site. It is known to catalyze the rearrangement of disulfide bonds of secretory proteins as well as being part of the prolyl-4-hydroxylase and it also represents the glycosylation site binding protein (LaMantia *et al.*, 1991).

A possible explanation for why some *vps* mutants are osmosensitive comes from the observation that most *vps* mutants in a *pep4-3* background are osmosensitive, while *pep4*-3 mutants as well as all but seven *vps* mutants in a *PEP4* background are not osmosensitive. An examination of the data of vacuolar protein mislocalization and maturation of PrA, PrB and CPY taken from Robinson *et al.*, 1988, seems to suggest that in all osmosensitive *vps* alleles hardly any CPY, PrA and PrB are matured or enzymatically active. Together with their extreme *vps* phenotype it seems possible that a component required for vacuole associated osmoregulation is partially mislocalized and the other fraction that is localized to the vacuole cannot be activated by PrA or a PrA activated secondary proteinase. Some ssv mutants accumulate the p2PrA, supporting this hypothesis. The osmosensitive phenotype of other *ssv* mutants isolated in this study which are not exhibiting extreme *vps* phenotypes or are maturation deficient, can be explained by that the selection for osmosensitive mutants specifically seclected for mutants which mislocalizes or inactivates the unknown osmoregulatory component but not other vacuolar proteins to the same extent. Another speculative alternative is that the vacuole has components that allow the immediate release of water from the vacuole into the cytoplasm to prevent precipitation of cytoplasmic proteins under altered osmotic conditions or water stress in the wild type before secondary measures such as regulation of water permeability by the plasma membrane can be taken. This is supported by the experimental evidence obtained in this study that salt-

stress is not immediately lethal in the wild type but is in all *ssv* mutants examined. These mutants could be defective in this pathway. Osmosensitive *vps* mutants could mislocalize these components to the cell surface and thus make this mechanism of immediate osmoregulation ineffective.

Alternatively, some mutants could have aquired an osmosensitive phenotype because of a mutation in the osmoadaptive glycerol regulatory pathway, or a mutation which inactivates an essential gene product under osmotic stress. So far unknown is the nature of osmoregulation and how the vacuole participates in it. The results obtained from the isolation of salt-sensitive mutants suggests that the vacuole actively participates in at least some osmoregulatory response. Other studies have addressed the adaptive osmoregulatory response, which enables *S. cerevisiae* to adapt to osmotically active medium facilitated by the accumulation of glycerol to compensate for the osmotically active surroundings (Blomberg and Adler, 1989). The same studies show that in an exponential culture only 0.02% of yeast cells are viable when exposed to 1.4 M NaCl, while stationary phase cultures are more osmotolerant as are cells that have been conditioned to low water stress. The results contradict the experimental results obtained in this study, because wild type yeasts cells remained viable after shift to YPD liquid medium supplemented with 1.5 M NaCl, and are able to grow albeit more slowly. It appears possible, though unlikely, that the low water potential will inhibit the growth of the majority of cells present in liquid culture and allows the small proportion of osmoresistant cells to actively grow, while the rest of the cells remains viable. The studies addressing the phenomenon of aquired osmotolerance (Blomberg and Adler, 1989) plated samples of different cultures directly onto YPD pour-plates supplemented with 1.4 M NaCl to test cells for osmotolerance, and only 0.02% of a exponential phase culture were able to form colonies. The difference in the experimental approach suggests that YPD + 1.4 M NaCl pour-agar plates impose a different osmotic stress onto individual yeast cells and cells might not be able to exert enough force to the surrounding to grow to colony size. Also the brief heating to 42°C before pouring might have influenced the experimental outcome. Alternatively, strain differences might account for the different findings.

Though glycerol plays an important part in a later response to osmotic changes in the surrounding (Blomberg and Adler, 1989), the same studies indicate that *de novo* protein synthesis is also required for the osmoadaptive response. This response is different from the heat shock induced trehalose accumulation in *Schizosaccharomyces pombe* which does not depend on *de novo* protein synthesis or hsp104 (De Virgilio *et al.*, 1990; De Virgilio *et al.*, 1991). The fact that unfavourable osmotic conditions such as 1.5 M NaCl present in the medium can terminally inactivate *ssv* mutants within seconds argues for the involvement of the vacuole in an immediate osmoregulatory mechanism that protects cells from cell death. The mutants isolated in this study are terminally inactivated by 1.5 M NaCl within 10 to 30 seconds. Other mutants were identified in the initial screening process which were inhibited in growth by the NaCl present in the medium but not killed, since they grew to small size colonies after 7 days. They were not characterized further to avoid unambiguities in cloning the corresponding genes by complementation of the salt-sensitive phenotype. These mutants are probably involved in a defect in secondary osmoregulatory responses since they remain viable. The fact that after prolonged periods of incubation (21 days) *ssv* mutants grown on equiosmolar concentrations of sorbitol will grow to normal colony size is indicative for their ability to adapt to osmotic conditions, and the initial lag observed may be characteristic for a defect in an immediate osmoregulatory response but since sorbitol has a membrane protecting function it might not kill the cells. Another reason for the differential effect is that the presence of either sodium or chloride ions can trigger the release or exchange of other ions directly or by changing the membrane potential. If the vacuole does not maintain its homeostatic function this process could lead to a depletion or intoxification of the cytoplasm followed by cell death. An osmosensitive mutant in *N. crassa* that is defficient in the accumulation of intracellular polyols is still able to increase its biomass although only by 10% when compared with the wild type (Ellis *et al.*, 1991). Although these data were obtained from a filamentous fungus it strongly suggests that mutants defective in the accumulation of glycerol will be viable to some degree under low water pressure conditions. Unfortunately, the nature of the *ssv* mutants does not permit to test for the accumulation of glycerol as adaptive

osmotic response since the mutations confer lethality under low water pressure if NaCl is used as an osmolyte. Evidence corroborating the above considerations that *ssv* mutants are not primarily defective in the adaptive osmoregulatory response come from a study in which a number of salt-sensitive mutants were isolated in *Zygosaccharomyces rouxii* (*Debaryomyces hansenii*) (Yagi and Toda, 1988). The mutants are deficient in their ability to synthesize glycerol under low water stress, maintain intracellular glycerol levels, take up glycerol from the medium or that had other defects. None of the mutants was salt-intolerant, unlike the *ssv* mutants which were isolated on the basis of salt-intolerance. Some bacteria accumulate K^+, amino acids and carbohydrates in their cytoplasm to prevent osmotic dehydration and to maintain turgor pressure (Elsheikh and Wood, 1990). The vacuole acts as store for K^+ and amino acids, and an immediate osmotic response also might involve the release of K^+ and amino acids to balance the osmotic gradient between cytoplasm and environment. A compromized vacuolar function can thus affect the immediate osmotic response of *S. cerevisiae*. Alternatively, the missorting of channels mediating an osmotic response to the plasma membrane might function properly under osmotic stress and drain the cytoplasm of osmoprotective components.

When interpreting the results presented in this study, there remains the possibility that genes other than in vacuolar function have been identified. Mutations in other genes unrelated to osmoregulation can have an osmosensitive phenotype, such as the *act1*-1 mutant, osmosensitive suppressor mutants, mutants in members of the translation machinery, and so forth. It has been argued that osmosensitivity in these mutations is related to temperature sensitivity in that a different environment (osmotic stress or temperature stress) can inactivate an essential gene product or that a change in osmotic conditions can promote misreading during translation in osmosensitive suppressor mutants (Singh, 1977). This model still does not account for the irreversibility of the salt-induced inactivation and the fact that mutants in 21 out of 23 complementation groups had vacuolar defects. Alternatively, defects in the above mentioned pathways could have secondary defects associated with vacuole biogenesis. The allelism or identity of the *ssv* alleles with known osmosensitive suppressors could not be tested.

Some vacuolar class A *ssv* mutants could be defective in the assembly or specificity of ion transport channels located in the plasma membrane. By analogy, the *HOL1*-1,-101 strain is hypersensitive to several ions due to a mutation altering the specificity of the *HOL1* encoded transporter protein (Gaber *et al.*, 1990). Finally, single mutations can alter the characteristics of voltage gated ion channels by changing ion specificity (Heinemann *et al.*, 1992), supporting the assumption that a point mutation could change specificities of ion channels.

Since four *ssv* mutants mislocalize vacuolar enzymes to a greater extent, the question arises whether new osmosensitive alleles of previously identified *vps* mutants were isolated. A genetic overlap based on the complementation of the osmosensitive phenotype was observable only between *ssv1*-2 and *vps1*. All other *vps* alleles which confer a salt-sensitive phenotype complement all *ssv* mutations. This has been confirmed by showing that in crosses between the 21 *ssv* complementation groups and the 47 *vps* complementation groups, only *ssv1*-2 and *vps1*, and *ssv9*-7 and *vps33* are identical based on the non-complementation of their *vps* phenotype. Other mutants isolated on the basis of different vacuolar defects, such as sensitivity to Ca^{2+}, showed extensive overlap with *vps* alleles (Klionsky *et al.*, 1990). Ca^{2+} sensitive *cls* mutants do not have an osmosensitive phenotype (Ohya *et al.*, 1986), which suggests that *ssv2*, *ssv3* and *ssv4* with Ca^{2+} sensitive alleles are not identical to *cls* genes. Several properties of some of the mutants isolated in this study suggest interesting new aspects of vacuole biogenesis. For example, gluconeogenic growth relies on some vacuolar function, and many mutants in aspects of vacuolar biogenesis are unable to grow on non-fermentable carbon sources. A possible explanation is that in some vacuolar mutants the vacuolar membrane protein Pho8p, which corresponds to fructose-2,6-bisphosphate 6-phosphatase, is not activated or its substrate fructose-2,6-bisphosphate (F2,6BP) cannot reach the vacuolar compartment. F2,6BP is produced in response to growth on glucose and disables gluconeogenesis by inhibiting fructose-1,6-bisphosphate 1-phosphatase and stimulating phosphofructokinase 1 to initiate glycolysis (Hers *et al.*, 1988). If the regulatory F2,6BP cannot be degraded it will arrest gluconeogenesis. Thus a hypothetical vacuolar defect not allowing the degradation of F2,6BP would prevent

gluconeogenesis and lastly growth on non-fermentable carbon sources. Another interesting though not desirable phenotype of exclusively all *ssv* mutants was their extremely low transformation efficiency. While electroporation was the only method these mutants could be transformed with, their efficiency was very low. One extreme was *ssv13*-1 which had a transformation efficiency of one transformant per 10 μg plasmid DNA. The nature of this defect is not known but could be associated with a vacuolar function since most class C *vps* mutants also transform badly (Banta *et al.*, 1988).

So far the only *SSV* gene products which have been identified are those for *SSV1*, *SSV9* and *SSV15* by virtue of their identity with *VPS1*, *VPS33* and *OSM2*, respectively, and *SSV7* which has been cloned and sequenced (Chapter 6). *VPS1* encodes a 80 kDa GTP binding protein related to mammalian Mx proteins (Rothman *et al.*, 1990), which recently has been shown to be of significant homology to the microtubule bundling-protein, dynamin (Yeh *et al.*, 1991). It appears to play an essential role in vacuolar protein sorting, membrane organization, growth at elevated temperature and spindle formation during mitosis. Evidence suggests that Vps1p is associated with some intermediate structure in the protein transport pathway, possibly the Golgi-apparatus, although its function and mechanism of action are still obscure. It appears likely that in the mutant the osmosensitive phenotype and fragmented vacuoles are secondary defects since the protein is not directly associated with the vacuole. Other small 21 kDa GTP binding proteins such as Ypt1p, Sec4p and the *rab* homologues, all closely related members of the *ras* super-family, are involved in directed vesicle traffic (Balch, 1990). However, the role of an 80 kDa GTP-binding protein in organelle biogenesis is unknown. Generally proteins binding and hydrolysing GTP are participants in molecular switch actions (Bourne, 1988). This can serve the purpose of signal amplification as is the case in trimeric GTPases or function in irreversibility or directionality of biochemical processes such as protein biosynthesis and vesicle mediated transport reactions (Bourne, 1988; Bollag and McCormick, 1991; Rodbell, 1992; Rothman and Orci, 1992). Vps1p binds and hydrolyzes GTP and associates with a membrane compartment which is probably identical with the Golgi apparatus (Rothman

et al., 1990; Raymond *et al.*, 1992). Both its membrane association and GTPase function are required for its sorting function and deletions in either domain results in quantitative secretion of vacuolar hydrolases (C.K. Raymond and T.H. Stevens, unpublished results). Since it is localized on the Golgi compartment there remains the possibility that it acts in mediating transport from the Golgi to the vacuole. A few possibilities appear likely: Vps1p could associate with the Golgi and allow budding of hypothetical transport intermediates to occur, similar to Sar1p on the ER membrane (d'Enfert *et al.*, 1991b). The fact that Vps1p remains associated with the Golgi is different to the situation from vesicle budding from the ER where Sar1p associates with Sec12p to enable budding but after this process is released following GTP hydrolysis. Vps1p could be a stationary molecular switch that signals the presence of receptor bound proteinases in the sorting compartment of the Golgi apparatus, and initiate the retrieval or budding mediated by other proteins. Since Vps1p is a dynamin homologue (Yeh *et al.*, 1991) the possibility remains that it performs a function in a cytoskeletal motor process (Vallee and Shpetner, 1990). Maybe it mediates the hypothetical budding or scission process from the Golgi sorting compartment for which GTP hydrolysis is required. The N-terminal domain is reminiscent of similar domains in myosin and kinesin representing a "force-producing" head (Obar *et al.*, 1991), which might function in the budding or scission of Golgi sorting vesicles, expecially since the bulk flow pathway is secrtion, and other budding processes might not rely on the force generated by lipid flow alone. The coexistence of constitutive Mx related proteins involved in intracellular transport and the mammalian Mx proteins induced in virally infected cells might suggest that the virally induced Mx proteins divert transport of viral particles to the lysosome rather than the cell surface (Arnheiter and Meier, 1990).

VPS33 encodes a hydrophilic protein of 691 amino acids with an ATP nucleotide binding site (Banta *et al.*, 1990; Wada *et al.*, 1990). Subcellular fractionation studies suggest that it is a cytoplasmic protein. The cellular function of this protein is currently not known, although certain alleles have an interesting phenotype in that they have a differential vacuolar segregation defect at 37°C. Most previously isolated *vps33* alleles are temperature sensitive for growth, independent of whether they have vacuoles or not

(Banta *et al.*, 1990). The *vps33* alleles isolated in this study are not temperature sensitive, although some do not have a vacuole (e.g. *ssv9*-7). This shows that the vacuole itself is not necessary to grow at elevated temperature contrary to evidence presented before (Banta *et al.*, 1988).

OSM2 appears to be identical to *ARO7*, which encodes chorismate mutase (Ball *et al.*, 1986). The mechanism of how an enzyme involved in aromatic amino acid biosynthesis can influence protein targeting and vacuole biogenesis remains obscure (Chapter 7). The nature of the other *ssv* mutants is subject to speculation. A number of genes, including some coding for vacuole associated proteins, cytoskeletal elements and proteins mediating vesicle budding and fusion are likely candidates.

In the following chapter an effort is made to elucidate the mechanism of osmoregulation in connection with vacuolar morphology or protein sorting in yeast by identifying and analysing the *ssv* genes and gene products. The present study already provides some preliminary evidence that the understanding of vacuole biogenesis is complicated by the fact that both vacuolar morphology and the sorting of vacuolar proteins is directly or indirectly under metabolic control. This phenomenon might also explain the large number of complementation groups involved in vacuolar protein sorting (Klionsky *et al.*, 1990; Raymond *et al.*, 1992), because they probably encode gene products required for the actual sorting process as well as mutations in the regulation of vacuole biogenesis or regulating the metabolic state of the cell. Evidence obtained from *Dictyostelium discoideum* indicates that lysosomal enzymes are secreted under starvation conditions (Dimond *et al.*, 1981). Maybe this is the case also in *S. cerevisiae*, and mutants affecting metabolic pathways could simulate starvation associated conditions and thus induce the secretion of vacuolar enzymes. Other enzymes in intermediate metabolism are associated with the cytoskeleton (Barnes *et al.*, 1992). One enzyme, glyceraldehyde 3-phosphate dehydrogenase in all three isoforms (GAPDH), is associated with microtubules. Furthermore, GAPDH bundles microtubules, suggesting a second role for the enzyme other than in catalyzing the metabolic step between glyceraldehyde 3-phosphate and 1,3-bisphosphoglycerate. Among several explanations, two seem plausible: 1. the enzyme is a true bifunctional

enzyme, and the functions present in the protein are independent from each other. 2. The enzyme attaches to the cytoskeleton to ensure distribution, or it is prevented from being degraded if disassociated. If the latter process is regulated by the metabolic state of the yeast cell the attachment could be part of a mechanism to regulate enzyme activity in response to metabolic changes analogous to glucose repession. Other metabolic enzymes, such as Arolp and Citlp also associate with microtubules (Barnes *et al.*, 1992). Lastly, the hydrolysis of GTP is associated with the molecular switch action of a number of cellular processes such as signal transmission, vesicle budding and fusion and protein biosynthesis (Bourne, 1988). The conformational change connected with this reaction often functions in affecting other proteins to change conformation or to make reactions irreversible. The GTPases might not be unique in performing a cellular function by GTP hydrolysis mediated conformational changes. Other enzymes change their conformation during catalysis also, and this change could be exploited by the cell to govern other processes, maybe subject to regulation by the metabolic state of the cell. A dissection of different functional domains could provide further knowledge about the nature and purpose of the interaction. The dual role of metabolic enzymes in metabolism as well as structural components is not new. Lens crystallin, the major protein in the optical lens, is a lactate dehydrogenase with metabolic function (Graeme and Piatigorsky, 1987; Hendiks *et al.*, 1988). Similarly, Gutlp and Gut2p, could directly or indirectly involve in vacuolar protein sorting either by associating or functionally participating with components of the vacuolar sorting pathway which is affected by the mutation and leads to missorting. Alternatively, the two enzymes when mutated disturb the metabolic equilibrium of the cell which directly or indirectly results in secretion of vacuolar enzymes which may or may not have a function *in vivo*. The two possibilities are difficult to distinguish at present. Directed studies addressing the function of Gutlp and Gut2p may provide an answer. Mammalian cells can localize otherwise lysosomal enzymes to the cell surface as a response to platelet derived growth factor (Prence *et al.*, 1990). This response involves the redistribution of the mannose 6-phosphate receptors such that the Golgi concentration of the receptor becomes limiting and thus causes the selective secretion of

a lysosomal protein. This protein has been identified as the major secreted protein (MEP) in transformed mouse fibroblasts strongly suggesting that carcinogenic transformation can result in missorting of proteins as a result of disturbances in signal transduction pathways. Golgi function in mammalian cells is affected by an oxysterol binding protein on ligand binding (Ridgway *et al.*, 1992). The hormonal response might alter the equilibrium of sorting or protein export from the Golgi. By analogy, vacuolar protein sorting might be subject to environmental stimuli in yeast. Whether or not similar mechanisms exists in yeast that allow the selective secretion of vacuolar proteins in response to environmental conditions is not known. Judging from the presence of different sorting paths for different vacuolar enzymes (Ammerer *et al.*, 1986; Paravicini *et al.*, 1992; Stevens *et al.*, 1986) this might be a likely possibility.

An *act1*-1 mutant has pleiotropic phenotypes including osmosensitivity and disrupted vacuoles (Novick and Botstein, 1985). The osmosensitive phenotype has been explained that osmotic stress destablized the mutant actin so that actin function cannot be carried out, similar to its temperature sensitivity. Three other possibilities theoretically exist. First, actin stabilizes vacuoles, and if destabilized, vacuoles fragment and perturb osmoregulatory functions. Second, mutations in actin result in a destabilized plasma membrane, which cannot adapt to changes in turgor and result in a collapse of the plasma membrane enclosed cell. Mammalian cells rely on the organization of F-actin to counteract osmotic changes in the surrounding (Kajstura and Reiss, 1989). Lastly, the mutant actin might be defective in the transmission of osmotically induced electrical signals and thus not initiate a perhaps linked osmoregulatory response. The last consideration is based on the finding that actin cables upon exposure to osmotic changes induce an electrical signal by changing its electrical potential and charge (Cantiello *et al.*, 1991). This change could affect the net membrane potential and thus open or close electrically regulated transmembrane channels. It has been suggested that the electrical properties of actin, which are also subject to changes following an electrical field change, might also change the structural status of actin polymerization and thus permitting structural remodelling of the plasma membrane or similar processes (Cantiello *et al.*, 1991).

It is believed that many proteins required for the Golgi to vacuole pathway have not been identified yet and the majority of proteins involved in this pathway are associated with the regulation of vacuolar protein sorting. Since there appear to be a large number of true *vps* mutants, other vacuolar mutants might be more difficult to isolate because the majority will affect sorting rather than the vacuolar function. Although this study provided a new approach to isolate vacuolar mutants, it is rather limited because up to date there is no possibility to discriminate between true vacuolar defects or associated defects due to alterations in the biochemical regulation of the organelle. Although successful in the isolation of sorting mutants, all present approaches to elucidate vacuolar function other than sorting and dissection of the vacuolar ATPase by reverse genetics have failed, and only isolated more sorting mutants. The *vac2* mutant so far seems to be an exception in that it is a true vacuolar segregation mutant which has no vacuolar sorting defect (Shaw and Wickner, 1991). The characterization of the *ssv* gene products will show whether they are directly involved in vacuole biogenesis and related pathways. There are numerous possibilities to extend the analysis of the *ssv* mutants and to examine the nature of their defect. For instance, their sensitivity or resistance to hygromycin B would show whether their defect involves the plasma membrane ATPase since vacuolar ATPase mutants are also osmosensitive (McCusker *et al.*, 1987). Also the monitoring of substances involved in osmoregulation, such as glycerol (Blomberg and Adler, 1989; Reed *et al.*, 1987), in response to osmotic stress would identify mutants that are defective in an osmoadaptive response rather than osmohomeostasis. The proposed role of K^+ in mediating osmoregulation was also not tested, because evidence obtained in other studies suggests that the K^+ status of the cell does not affect the osmoadaptive response in *S. cerevisiae* (Meikle *et al.*, 1991). The exchange of ions could be measured, or the influx of Na^+ or Cl^- monitored. It was decided against an extensive analysis of the *ssv* mutants because lastly it will always be difficult to jugdge whether any of the described defects is a primary defect or a consequence of another defect. The isolation and characterization of the corresponding gene products appeared more profitable, lastly because the identification and analysis of gene products participating in osmoregulation and/or

vacuole biogenesis allows a more detailed study of their cellular function. This was attempted by isolating a gene which complements the *ssv7*-1 mutation (Chapter 6).

CHAPTER 5

CONSTRUCTION OF A PLASMID BASED YEAST GENOMIC DNA LIBRARY

5.1. Introduction

The amenability of *S. cerevisiae* to genetic as well as molecular biological methodology permits the isolation of genes corresponding to mutant alleles by complementation of the mutant phenotype. Analogous to complementation of a mutation by a wild type copy of the same gene in heterozygous diploids, a recessive mutation in a yeast gene can functionally be complemented by a wild type copy of the gene present on a plasmid if the mutation confers a selectable phenotype. The cloning by complementation approach is feasible because the size of the yeast genome of only 15,000 kb permits the construction of a genomic plasmid library, and also *S. cerevisiae* can easily be transformed with plasmid DNA by diverse means. Thus, a relatively small number of clones has to be screened. This reasoning has led to the construction of several genomic DNA libraries in yeast shuttle vectors that simultaneously allow the maintenance of the plasmid library in *E. coli*, as well as the introduction and stable maintenance of library clones in *S. cerevisiae* (Nasmyth and Reed, 1980; Carlson and Bostein, 1982; Rose *et al.*, 1987). The earlier yeast genomic DNA libraries were constructed by ligation of random genomic DNA fragments into yeast shuttle vectors based on the *ARS1* element for autonomous replication (Nasmyth and Reed, 1980), or into vectors derived from the 2μ plasmid (Carlson and Botstein, 1982). However, the shuttle vectors are maintained in high copy number in yeast, which can lead to problems if the gene product is toxic when overexpressed (Rose and Fink, 1987). Also, overexpression of genes other than the mutated gene can lead to a suppression of the defect used for selection (Kuo and Campbell, 1983; MacKay, 1983; Pringle *et al.*, 1984; Bender and Pringle, 1989), especially if the gene products interact with each other

(Adams and Botstein, 1989; Novick *et al.*, 1989). Although useful for the identification of genes whose gene products directly interact or are involved in signal transduction pathways, the isolation of a clone by complementation as well as multicopy suppression can introduce ambiguity into the identification of the gene. Another limitation of the use of *ARS1* and 2μ based shuttle vectors is their instability, which will introduce complications when analysing a particular phenotype, because individual yeast cells will have different concentrations of the complementing gene product - if any - present. These considerations have led to the construction of a YCp50 based genomic DNA plasmid library (Rose, 1987; Rose *et al.*, 1987), where random fragments of yeast DNA were inserted into the centromeric shuttle vector YCp50, which is present in low copy number (1 to 2 per cell) and mitotically stable (less than 1% loss per generation per cell). However, genes toxic to *E. coli*, as well as genes proximal to centromeres will be underepresented, because of the high copy number of the pBR322 derived shuttle vector in the bacterial host, and the frequent breakage of dicentric plasmids, respectively.

A common finding in *S. cerevisiae* is that different strains of the same species can differ widely in the restriction map of identical genes (Rose, 1987), and thus genetic studies as for instance restriction map verification of single step gene disruptions with the same gene from a different host strain can lead to ambiguities. It was therefore desirable in this study to construct a yeast genomic DNA library from the same strain from which the *ssv* mutants were isolated. Another advantage of constructing a new library rather than amplification of an existing library is that the loss of rare clones which have a selection disadvantage during bacterial growth can be minimized (Vogeli and Kaytes, 1987).

The above considerations led to the construction of a *S. cerevisiae* SEY6210 genomic DNA library in YCp50, principally as described in Rose *et al.*, 1987, which was subsequently used in attempts to clone the genes corresponding to the *ssv* mutant alleles isolated in this work.

The isolation of yeast genes by complementation from a plasmid based library requires an efficient method of transformation. Several such methods have been

published, which are either based on the transformation of yeast sphaeroplasts (Beggs, 1978), the use of Li^+ ions (Ito *et al.*, 1983), or electroporation of DNA into the cell (Meilhoc *et al.*, 1990). Although a very efficient method, the transformation of sphaeroplasts brings about some problems which make it undesirable for transforming a library into yeast. For example, co-transformation of several plasmids into the same cell is a common phenomenon that can make the identification of positively complementing clones by curing the transformant of the plasmid difficult (Rose, 1987; Rose *et al.*, 1987). Secondly, some properties of the *ssv* mutants indicated potential difficulties in the choice of the optimal transformation method. The *ssv* mutants described in this study were isolated on the basis of sensitivity to high osmolarity of the growth medium. This also decides against using the sphaeroplast transformation method and also some electroporation protocols, because both require the addition of an osmotic stabilizer (1.2 M sorbitol) immediately before or after transformation (BioRad Electroporator manual). Another property of most *ssv* and also some *vps* mutants is the grossly reduced transformation efficiency, which might make the Li^+ based methods unsuitable because of their lower transformation efficiency compared to the sphaeroplast method (Ito *et al.*, 1983). These considerations led to a trial of all available methods on one of the *ssv* mutants and the SEY6210 wild type to find the most efficient transformation protocol.

5.2. Results

5.2.1. Isolation of SEY6210 Genomic DNA

SEY6210 derived genomic DNA was isolated as described in Materials and Methods according to the method desribed in Rose *et al.*, 1987, except that the preparative sucrose gradient step to separate genomic DNA from mitochondrial DNA was omitted to avoid unnecessary shearing of the DNA during the experimental procedure. A total of 1260 µg DNA was recovered from a 1 L culture, of which the majority migrated to a size of approximately 50 to 60 kb on a 0.4% agarose gel (Figure 5.1).

5.2.2. Plasmid Bank Construction

The SEY6210 genomic DNA was partially digested with *Sau* 3A using different concentrations of the enzyme to establish ideal conditions for preparative digests to yield genomic DNA fractions over a range of 10 to 30 kb in size. It was digested with 2 U/μg DNA to 0.015 U/μg DNA at 37°C for 60 minutes and the digests separated on a 0.4% agarose gel. As the highest fluorescence of the 10 to 30 kb region of the gel was reached at a *Sau* 3A concentration of 0.015 U/μg SEY6210 DNA (Figure 5.1), half the enzyme concentration of the same batch (0.008 U/μg DNA) was used for a preparative digest to yield the maximum number of molecules in that size range, because the intensity of fluorescence is related to the mass distribution of DNA, not the size distribution (Maniatis *et al.*, 1982).

500 μg SEY6210 genomic DNA was digested with 0.008 U/μg DNA for 60 minutes, and after phenol:chloroform extraction and subsequent ethanol precipitation, the partially digested DNA was separated according to size on a preparative 10 to 40% sucrose gradient. 0.5 mL fractions were collected from the top and every third fraction was screened for the size of the separated DNA (Figure 5.2). The size of DNA was related to the fraction number (Figure 5.3), and fractions 36 to 41 were pooled to yield size fragments between 10 to 15 kb, 42 to 47 to yield fragments of 15 to 20 kb, and fractions 48 to 51 for 20 to 30 kb fragments. The pooled fractions were dialyzed against TE buffer and concentrated by ethanol precipitation.

Bam HI cut and dephosphorylated YCp50 DNA (see Figure 5.4 for the plasmid map) was combined with size-fractionated SEY6210 DNA in a 2:1 mass ratio of vector to insert, and ligation of the 10 to 15 kb DNA was carried out at 30 μg/mL, the 15 to 20 kb DNA at 22.5 μg/mL, and of the 20 to 30 kb DNA at 15 μg/mL total DNA concentration. The ligations were transformed into *E. coli* for amplification of the library.

5.2.3. Optimal Conditions for Tranformation of the Gene Bank into *E. coli*

To avoid loss of rare clones underepresented in the SEY6210 genomic DNA plasmid bank, it was sought to employ the most efficient method of transformation with the recommended host strain HB101 at an ideal plasmid DNA concentration.

The most efficient method of transformation of *E. coli* with plasmid DNA is that described by Kushner *et al.*, 1978, which routinely transforms at a plasmid probability of 3.0×10^{-4} for strain HB101, the plasmid probability being defined as the number of transformants at 2.0×10^{8} cells divided by the number of plasmids present (Hanahan, 1983). Although JM83 transforms at a more efficient ratio than the *rec*A$^-$ HB101, it was not used for maintaining the plasmid bank, because recombination of highly repetitive yeast genomic DNA sequences was a common occurrence (results not shown). Using different concentrations of YCp50 vector DNA, ranging from 1 ng to 500 ng per transformation, it was established that 10 ng DNA per transformation gave the best plasmid probability (3×10^{-4}) of YCp50 with HB101 as host (Figure 5.5).

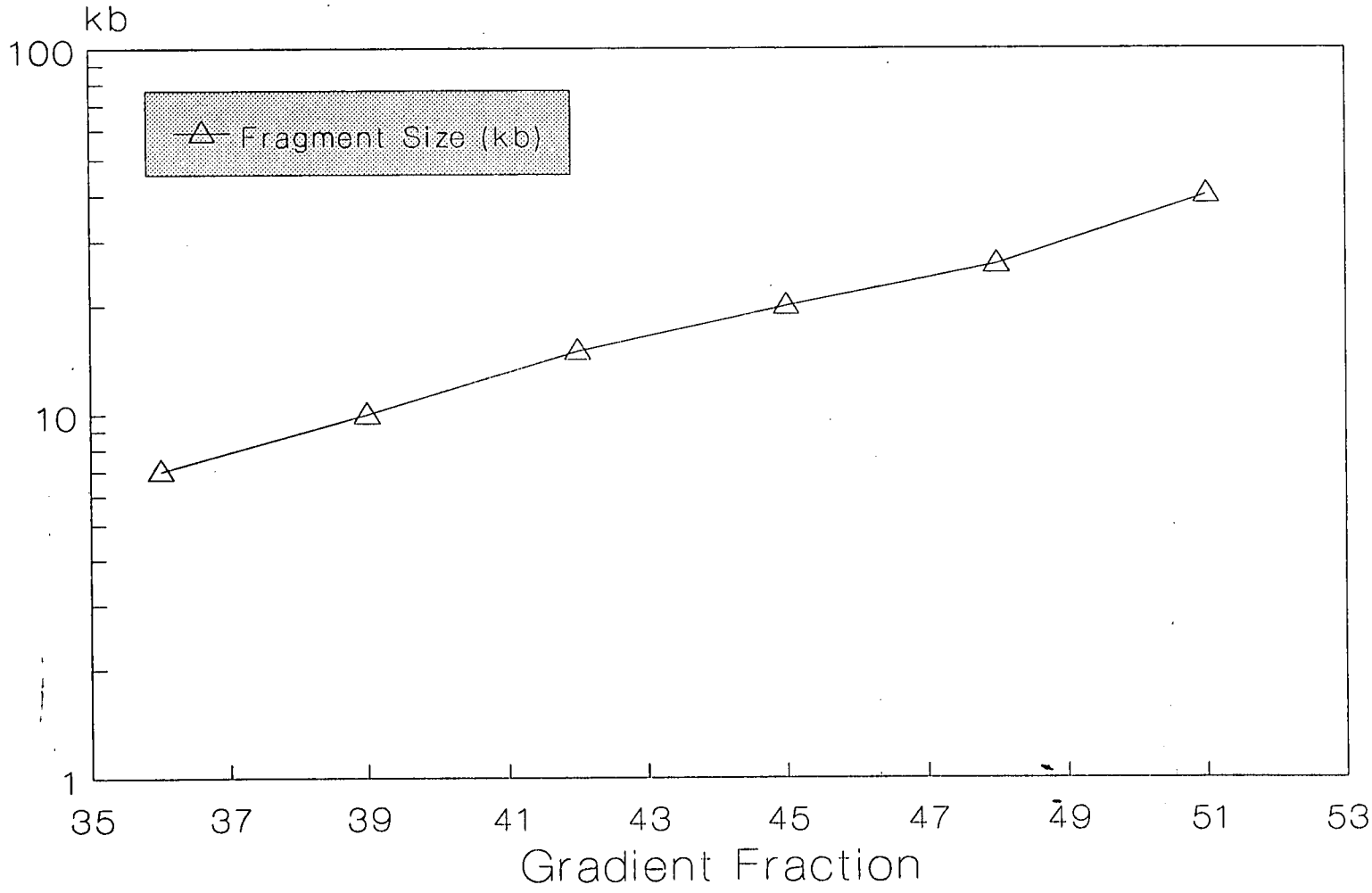
Figure 5.1: Partial digestion of SEY6210 genomic DNA (9) with *Sau* 3A to establish the restriction enzyme concentration needed to create a maximum number of fragments between 10 and 30 kb. 10 μg DNA was digested with varying concentrations of *Sau* 3A for 60 minutes and electrophoresed in a 0.4% agarose gel. The enzyme concentrations were 2 U/μg DNA (1), 1 U/μg DNA (2), 0.5 U/μg DNA (3), 0.25 U/μg DNA (4), 0.12 U/μg DNA (5), 0.06 U/μg DNA (6), 0.03 U/μg DNA (7) and 0.015 U/μg DNA (8). Molecular size markers were λ (*Eco* RI) (10), λ (*Pst* I) (11) and λ DNA (12). The largest restriction fragment of each marker correspond to 21.2 kb (10), 14.1 kb (11) and 48 kb (12).


1 2 3 4 5 6 7 8 9 10 11 12

Figure 5.2: Fractions recovered from the sucrose gradient. Fractions 33 (1), 36 (2), 39 (3), 42 (4), 45 (5), 48 (6) and 51 (7) were electrophoresed in a 0.4% agarose gel. Molecular size markers were λ (*Eco* RI) (10), λ (*Pst* I) (11) and λ DNA (12). The largest restriction fragment of each marker correspond to 21.2 kb (9), 14.1 kb (10) and 48 kb (11). The region of brightest fluorescence was extrapolated to determine the average molecular size of the fraction. Data were presented in graphic form (Figure 5.3).


1 2 3 4 5 6 7 9 10 11

Figure 5.3: Average fragment size (kb) of *Sau* 3A partially digested SEY6210 DNA after fractionation on a 10 to 40% sucrose gradient. The average fragment size was determined by relating the region of greatest fluorescence in Figure 5.2 to the relative distance migrated on the agarose gel against standards of known size.


kb
100
10
1
Fragment Size (kb)
35
37
39
41
43
45
47
49
51
53
Gradient Fraction

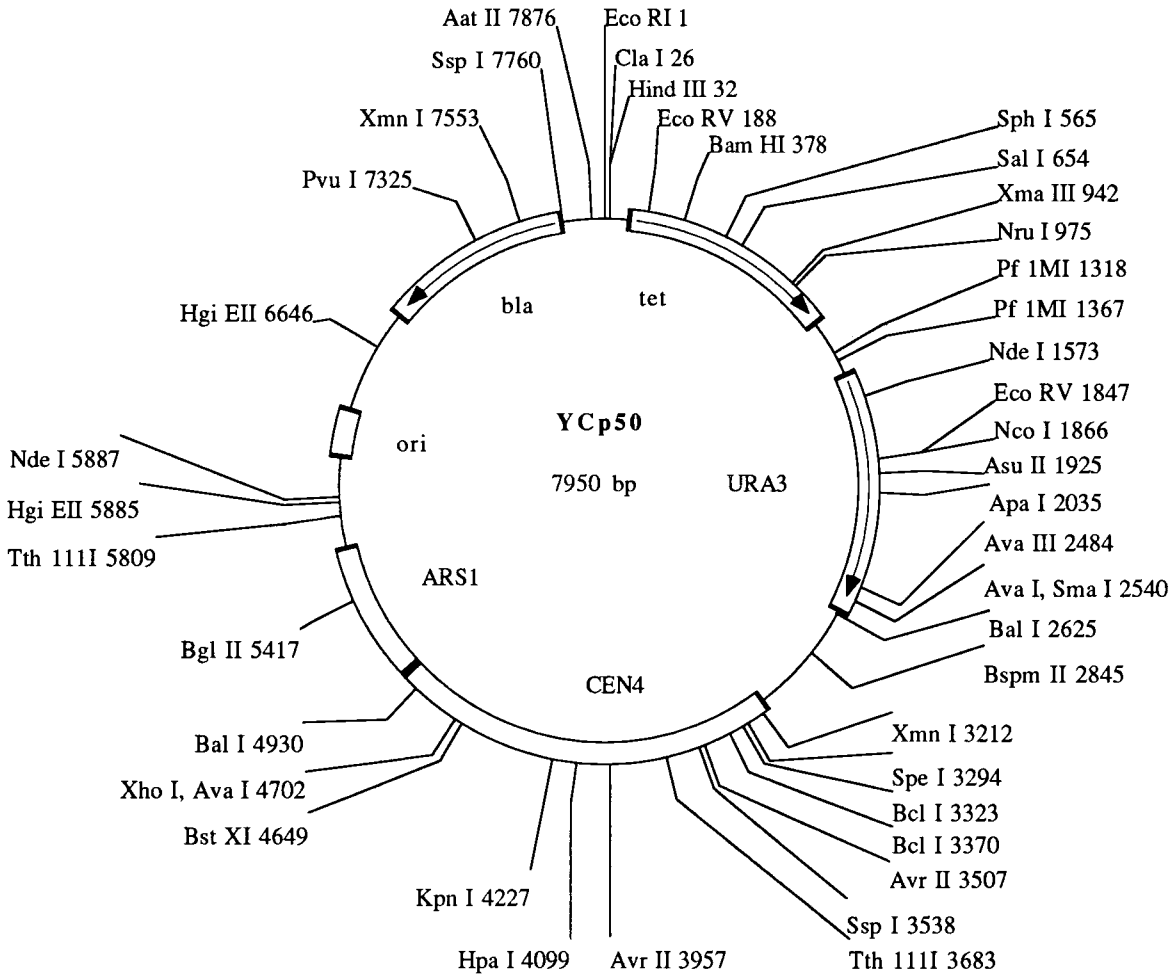
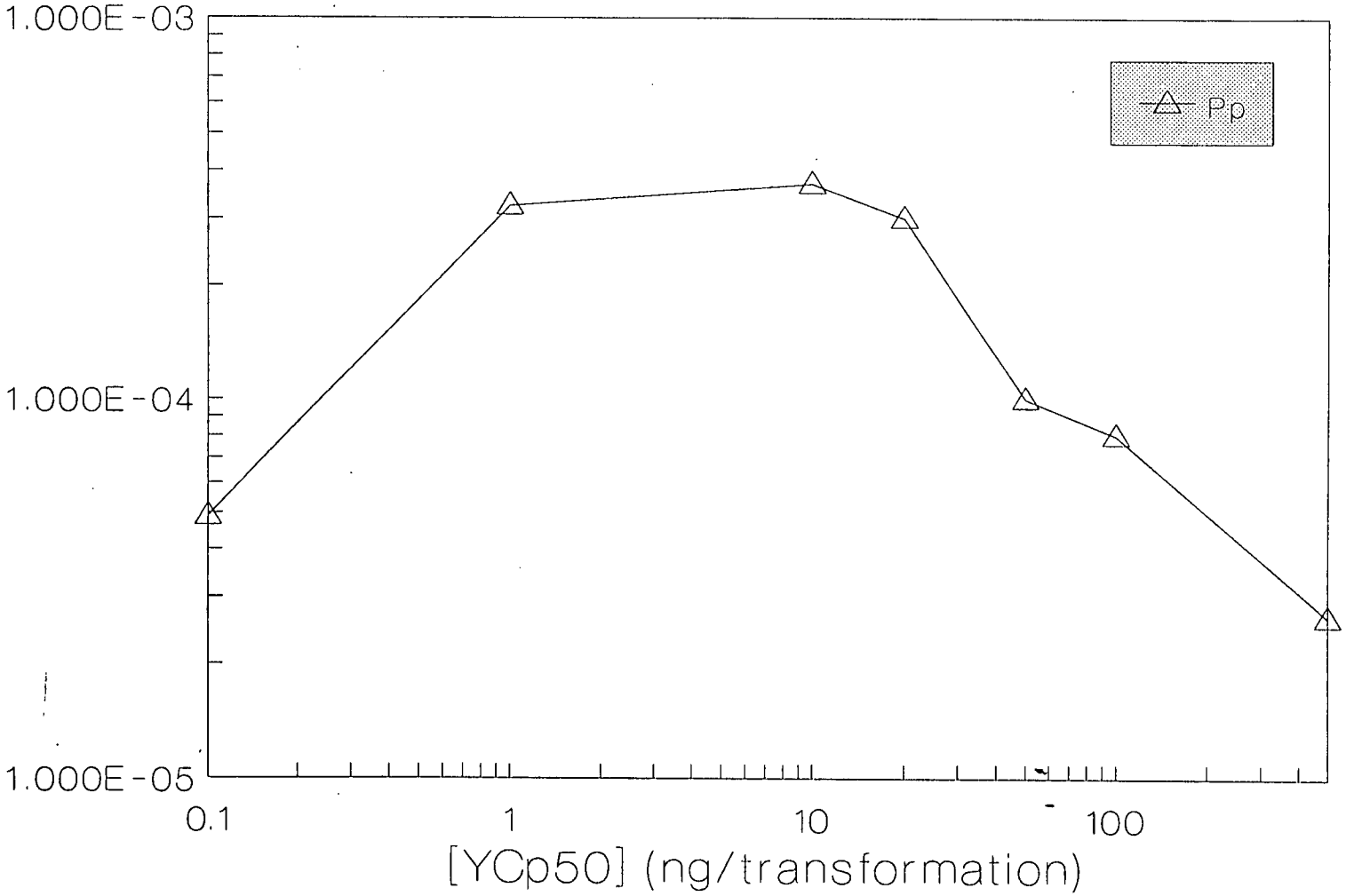

Aat II 7876
Eco RI 1
Ssp I 7760
Cla I 26
Hind III 32
Xmn I 7553
Eco RV 188
Bam HI 378
Sph I 565
Sal I 654
Pvu I 7325
Xma III 942
Nru I 975
Pf 1MI 1318
Hgi EII 6646
bla
tet
Pf 1MI 1367
Nde I 1573
YCp50
Eco RV 1847
Nco I 1866
ori
Nde I 5887
7950 bp
URA3
Asu II 1925
Hgi EII 5885
Apa I 2035
Tth 111I 5809
Ava III 2484
ARS1
Ava I, Sma I 2540
Bal I 2625
Bgl II 5417
Bspm II 2845
CEN4
Xmn I 3212
Bal I 4930
Spe I 3294
Xho I, Ava I 4702
Bcl I 3323
Bst XI 4649
Bcl I 3370
Avr II 3507
Kpn I 4227
Ssp I 3538
Hpa I 4099
Avr II 3957
Tth 111I 3683

Figure 5.4: Restriction map of YCp50.

Figure 5.5: The probability of transformation (Pp) of HB101 with YCp50 is drawn as a function of the absolute amount of YCp50 DNA present per tranformation of 2 $x10^8$ competent cells. The Pp ratio is defined as the ratio of transformants at a total cell concentration of 2 x 10^8 cells/mL divided by the number of plasmids present, assuming that 1 ng of an 8 kb plasmid is equivalent to 1 x 10^8 plasmid molecules (Hanahan, 1983). The curve shows that an optimal Pp ratio of approximately 3 x 10^{-4} is reached at a plasmid concentration of about 10 ng/transformation.


1.000E-03
1.000E-04
1.000E-05
0.1
1
10
100
Pp
[YCp50] (ng/transformation)

5.2.4. Maintenance of the Gene Bank in HB101

The plasmid bank was transformed into ultra-competent HB101 at the ideal DNA concentration of 10 ng DNA/transformation, and transformants were selected on Luria broth supplemented with ampicillin. All transformants from each ligation were harvested by washing off the transformants on each plate with 1 mL sterile Luria broth, which were then pooled and centrifuged. Pellets were resuspended in 1 mL sterile 50% (v/v) glycerol and stored at -84^{o}C for long term storage. Aliquots of each bank were diluted, and the number of insert containing YCp50 plasmids was determined. Overall 70% of all clones in the library did contain inserts significantly larger than the YCp50 host vector as judged by electrophoresis of plasmid mini-preps isolated from 20 clones for each library pool (results not shown). A total of four library pools for each size class was produced (table 5.1), where the estimated size class was based on the electrophoretic mobility of SEY6210 genomic DNA fragments after fractionation on a preparative sucrose gradient. The overall number of independent clones was about 35,500 in 12 separate libraries. Large scale plasmid preparations were made from pooled cells after diluting the cells to 2 x 10^{7} cells/mL in Luria broth supplemented with ampicillin and growing the culture to saturation.

Library	Estimated Size Class	No. of Independent Clones	Volume equivalent to 2 x 10^7 clones
A1	10 - 15 kb	7119	15 µL
A2	10 - 15 kb	3701	5 µL
A3	10 - 15 kb	3066	5 µL
A4	10 - 15 kb	2629	5 µL
B1	15 - 20 kb	2199	3 µL
B2	15 - 20 kb	2233	2 µL
B3	15 - 20 kb	2479	2 µL
B4	15 - 20 kb	4815	13 µL
C1	20 - 30 kb	1400	3 µL
C2	20 - 30 kb	1470	3 µL
C3	20 - 30 kb	2480	8 µL
C4	20 - 30 kb	1570	25 µL

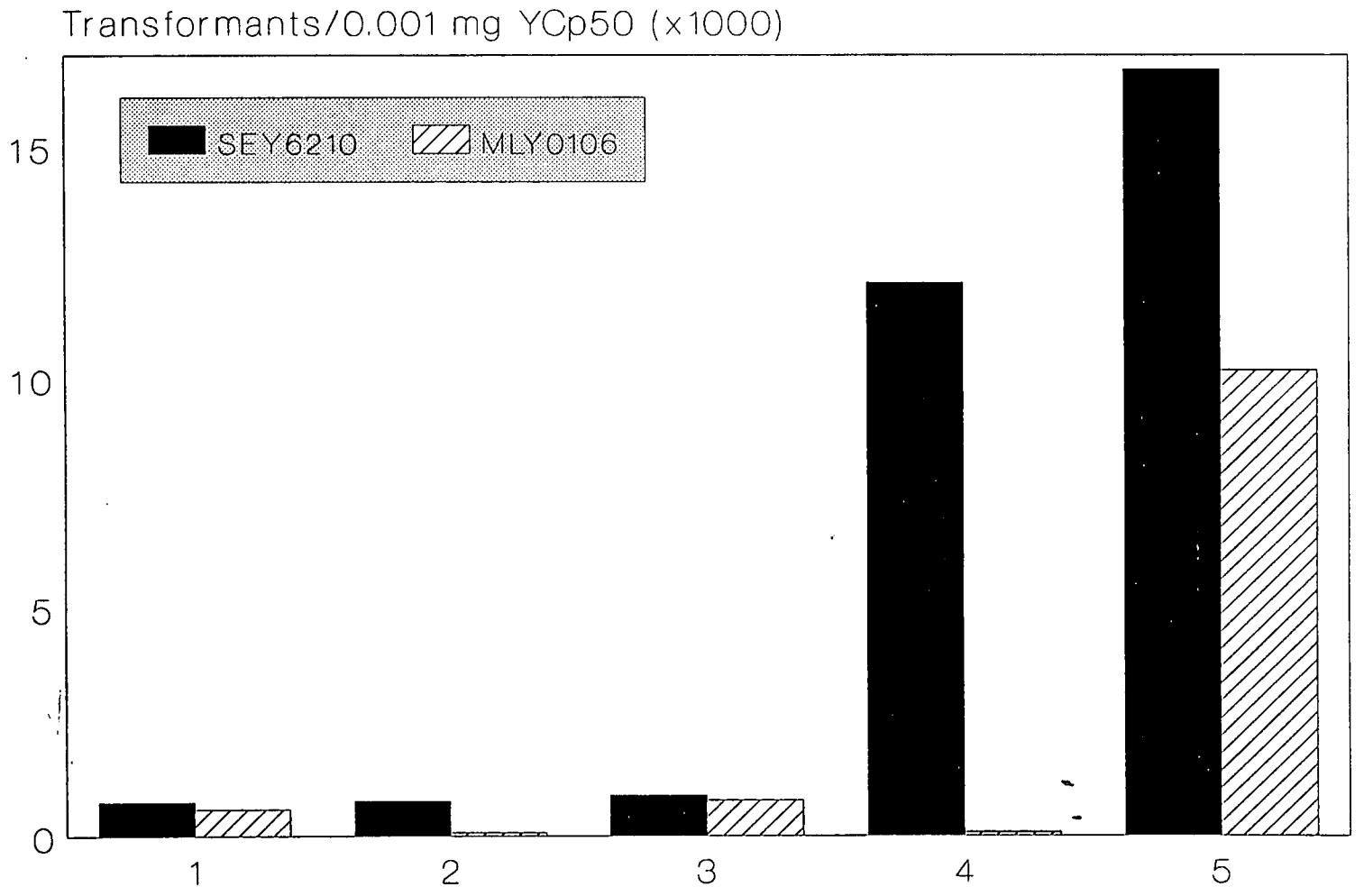
Table 5.1: SEY6210 genomic DNA plasmid bank structure. The total number of independent clones represented the number of independent HB101 transformants which harbour insert containing plasmids. The estimated size class was based on the electrophoretic mobility of SEY6210 DNA fragments after fractionation on a preparative sucrose gradient. The absolute insert size as determined by restriction mapping of some average size clones represented the lower range of the corresponding size class (data not shown).

5.2.5. Efficiency of Transformation Protocols for *S. cerevisiae*

Strains SEY6210 and MLY0106 (*ssv1*-2) were processed in accordance with the three lithium acetate protocols, the sphaeroplast transformation protocol and the electroporation protocol as described in Materials and Methods. YCp50 DNA was used to transform the strains at concentrations that were thought to be optimal by the developers of each transformation protocol. Transformants were selected on suitable medium lacking uracil but supplemented with all other required amino acids.

A comparison of the five different, commonly used methods shows that electroporation of dithiothreitol treated intact yeast cells (Meilhoc *et al.*, 1990) yields a higher transformation efficiency for 1 µg YCp50 DNA for both the wild type as well as the *ssv1*-2 allele (Figure 5.6). In fact, this method appears to be the only method to transform the *ssv1*-2 allele with YCp50 DNA with high efficiency. Elecroporation yields about 1.5 times more transformants/µg DNA in the wild type than the sphaeroplast transformation (Beggs, 1978), and 22.6 times as many transformants/µg DNA than the lithium acetate based protocols. For the *ssv1*-2 allele, the electroporation is 40% less efficient than for the parental wild type strain, but still 14.3 times more efficient than the most efficient lithium acetate protocol after U. Certa. The *ssv1*-2 mutant strain MLY0106 could not be transformed with the sphaeroplast transformation method, probably because the sorbitol required in the regeneration medium inhibits growth of the osmosensitive mutant strain.

Figure 5.6: Efficiency of transformation of the wild type SEY6210 and a representative *ssv* mutant, MLY0106 (*ssv1*-2), with different transformation protocols. 1: Lithium acetate method (Ito *et al.*, 1983); 2: Improved lithium acetate protocol (M.D. Watson, personal communication); 3: Improved lithium acetate method (U. Certa, personal communication); 4: Sphaeroplast transformation protocol (Beggs, 1978); 5: Electroporation of dithiothreitol treated intact yeast cells (Meilhoc *et al.*, 1990). The transformation efficiency is expressed in absolute numbers of transformants obtained for each μg YCp50 transformed in oder to choose the most efficient method in terms of use of plasmid DNA.


Transformants/0.001 mg YCp50 (x1000)
SEY6210
MLY0106
15
10
5
0
1
2
3
4
5

5.3. Discussion

High molecular weight SEY6210 DNA was partially digested with *Sau* 3A and fractionated by size on a preparative sucrose gradient. Different size classes of SEY6210 yeast DNA were collected and ligated into the *Bam* HI site of YCp50 to yield 12 independent libraries (Table 5.1). Assuming an average size of the *S. cerevisiae* genome of 15,000 kb (Lauer *et al.*, 1977), each individual library represents between 1.7 and 4.7 times the yeast genome, although the absolute number of genomic clones will be smaller because of the presence of mitochondrial DNA sequences. It was decided to maintain separate libraries according to genomic insert size classes rather than pooling all plasmid clones into a larger library to reduce bias during amplification in *E. coli* due to different growth properties of cells harbouring plasmids of different size (Rose *et al.*, 1987). Additionally, a library containing plasmids of widely different size are expected to select for the smaller plasmids in a population, since larger plasmids are transformed less efficiently into both *E. coli* and *S. cerevisiae*. The screening of several independent libraries will also ensure that plasmid isolates are of independent origin, rather than identical clones of the same plasmid (Rose *et al.*, 1987).

Although a centromere based plasmid library will avoid the isolation of multicopy suppressor alleles of a given mutant allele, the *SEC4* gene was able to suppress certain *sec15*, *sec8* and *sec2* alleles if inserted in YCp50 (Salminen and Novick, 1987). The identity of the library copy with the originally isolated mutant allele has to be confirmed in each case by integration mapping. This can be achieved by cloning the library isolate into YIp5 which will integrate next to the chromosomal copy, and subsequent Southern analysis to screen for correct integration of the wild type copy next to the chromosomal allele. If both mutant allele and library allele are identical, the mutant phenotype will cosegregate with the auxotrophy marker of the YIp5 vector (Salminen and Novick, 1987). Gene disruption analysis ("null alleles") and subsequent analysis of the associated phenotype can provide further evidence of identity of the complementing gene as the one which contains the mutation (Rose, 1987).

The YCp50 based library constructed in this work is comparable to the one described by Rose *et al.*, 1987, except that SEY6210 DNA rather than GRF88 DNA was used for the construction. Three genes, *SSV7* (see Chapter 6), *SSV9* and a *ts* suppressor of the *ssv9*-1 allele (M. Latterich, unpublished results), were isolated from the library up to date. All isolates were present at an approximate frequency of 0.3%, which lies within the estimated frequency of 0 to 0.4% of acquiring complementing plasmids from any single library pool (Rose *et al.*, 1987). So far no complementing clones were isolated from this library for *ssv3*-1, *ssv5*-2, *ssv10*-1, and *ssv13*-1 (M. Latterich, unpublished results). The failure to isolate complementing clones is most probably due to the very low transformation efficiency of the *ssv* mutant alleles mentioned above, rather than the absence of the corresponding genes from the library. The library described here has also been successfully screened by hybridization to isolate the *OSM1* gene (Ma, 1991).

The above findings suggest that electroporation is the method of choice when transforming the YCp50 based library into *ssv* mutant strains. Although far more efficient than other transformation methods, some *ssv* alleles still failed to be transformed efficiently. For the *ssv13*-1 allele, for example, only two transformants were obtained after transforming a total of 20 µg library DNA into the strain. For some obscure reason most *ssv* alleles and also most vacuolar class C *vps* alleles transform magnitudes less efficiently than the corresponding wild type strains, even after backcrossing (S.D. Emr, personal communication; M. Latterich, unpublished data). The reason for this phenomenon is yet unknown, but it appears that some of the *SSV* gene products are required for recovery of the transformed strains rather than for the efficiency of transformation. In case of strains carrying the *ssv7*-1 mutant allele, the mutant strain has a strongly reduced apparent transformation efficiency with eqimolar amounts of YCp50, than with the *SSV7* conferring plasmid pMLY36, which reaches almost the transformation efficiency of the wild type with YCp50 (M. Latterich, results not shown).

A way to circumvent the problem of direct gene bank transformation into low frequency transformable *ssv* alleles would be to employ the strategy used by Novick *et*

al., 1989, which was devised by J. Rhine, University of California, Berkeley, USA (unpublished data). In brief, a *kar1* strain with high transformation efficiency is transformed with the gene bank. The library transformants then are replica plated onto lawns of the *ssv* strain to be complemented with the libray on YPD plates. The strain to be transformed has to be of opposite mating type as the *kar1* mutant strain and has to have at least one drug resistance marker (e.g. CAN1 or CYH1). Cytoductants can be selected on medium lacking uracil (YCp50 based library) but supplemented with canavanine and/or cycloheximide (both resistance markers are recessive in heterozygous diploids). Colonies growing under these conditions are probably cytoductants of the recipient strain, since the *kar1* strain is more likely to transfer the plasmid than the nucleus during mating. The library cytoductants can then be screened for complementation of the mutant phenotype analogous to library transformants. One possible dawback this method has is that most *ssv* strains that transform poorly also show a reduced ability to mate with strains of the opposite mating type.

CHAPTER 6

CHARACTERIZATION OF THE *SSV7* GENE

6.1. Introduction

The feasability of cloning yeast genes by complementation has led to progress in understanding the molecular basis of many cellular processess, the most prominent being the cell cycle (Forsberg and Nurse, 1991) and the secretory pathway (Schekman, 1985). The analysis of enzymes in biochemical pathways was greatly facilitated by identifying the structural and regulatory genes involved and allowed a manipulation of genes and their products. The same reverse genetic approach has been employed by a number of researchers to dissect vacuole function (reviewed in Klionsky *et al.*, 1990; Raymond *et al.*, 1992; see also Chapter 4). Lastly the isolation and characterization of salt-sensitive vacuolar mutants (*ssv*) was intended to facilitate the cloning and molecular characterization of genes and their products participating in vacuole biogenesis and to examine the validity of the previously voiced hypothesis of vacuole mediated osmoregulation (Banta *et al.*, 1988; Klionsky *et al.*, 1990). While the isolation of osmosensitive vacuolar mutants was successful (Latterich and Watson, 1991; Chapter 4), a preliminary phenotypic analysis did not provide any evidence in favour or against vacuole mediated osmoregulation. Considering previous studies which stressed the overlapping function of vacuolar processes (reviewed in Klionsky *et al.*, 1990) and the fact that many differing isolation protocols resulted in the isolation of identical vacuolar sorting mutants, it appears to be necessary to examine the role of gene products functioning in vacuole biogenesis at the molecular level. Complementation analysis of the *ssv* mutations showed that excepting three complementation groups none were identical to other vacuolar protein sorting mutants (Chapter 4).

ssv mutants of different complementation groups were transformed with a plasmid based yeast genomic DNA library (Chapter 5), and genes complementing three different

mutants were identified (M. Latterich, unpublished results). In the following the molecular cloning and preliminary characterization of the *SSV7* gene and the corresponding *ssv7*-1 mutant are described.

Although a strain carrying the *ssv7*-1 mutant allele only has a moderate sorting defect in that about 20% of the vacuolar protein carboxypeptidase Y is mislocalized to the cell surface and it had a class B vacuolar phenotype, it was the first mutant gene that was cloned by complementation. Subsequent analysis of the complementing gene and a more detailed phenotypic characterization of the original *ssv7*-1 mutant strain provided many promising observations that may lead to an understanding of the nature of key cellular processes such as nuclear assembly and the regulation of the cell cycle.

6.2. Results

6.2.1. Morphological Features Conferred by the *ssv7*-1 Allele

A routine analysis of the *ssv7*-1 mutant strain by fluorescence microscopy indicated that apart from a class B vacuolar morphology it had a nuclear defect judged by DAPI staining of fixed cells (Figure 6.1). While wild type cells have a very distinct and confined DAPI fluorescence pattern representing the nucleus, and a punctate fluorescence pattern in the periphery of the cell near to the plasma membrane representing mitochondrial DNA, *ssv7*-1 mutants had slightly larger confined loci of fluorescence near the plasma membrane probably representing exaggerated mitochondria. The distinct nuclear fluorescence was absent in these mutants and the DAPI fluorescence was more diffuse and evenly distributed throughout the cytoplasm. *ssv7*-1 cells were approximately twice to three times as large as wild type cells, which could also explain the fact that the mitochondria in these cells appeared larger.

Figure 6.1: Light and fluorescence microscopical analysis of fixed MLY1511 (*ssv7*-1) (A-C) compared to fixed SEY6210 (wild type) (D-F). Normarski contrast microscopy (A and D) revealed that MLY1511 had approximately two to four times larger cells (A) than the isogenic wild type (D) and also a highly disrupted vacuole typical for vacuolar class B mutants. The latter was confirmed by indirect-immunofluorescence labelling of fixed sphaeroplasts with anti-Pho8p antiserum, which localizes the vacuolar membrane protein (C and F). MLY1511 showed fluorescence localization of several small vacuoles (C), which in the wild type was located around a large vacuole (F). DAPI staining revealed that MLY1511 has a diffuse cytoplasmic staining pattern and a punctate pattern near the plasma membrane probably representing exaggerated mitochondria (B), unlike the wild type which has a defined highly fluorescing nuclear staining patterns and a small punctate mitochondrial fluorescence (E). 0.5 cm equivalent to 1 μm.

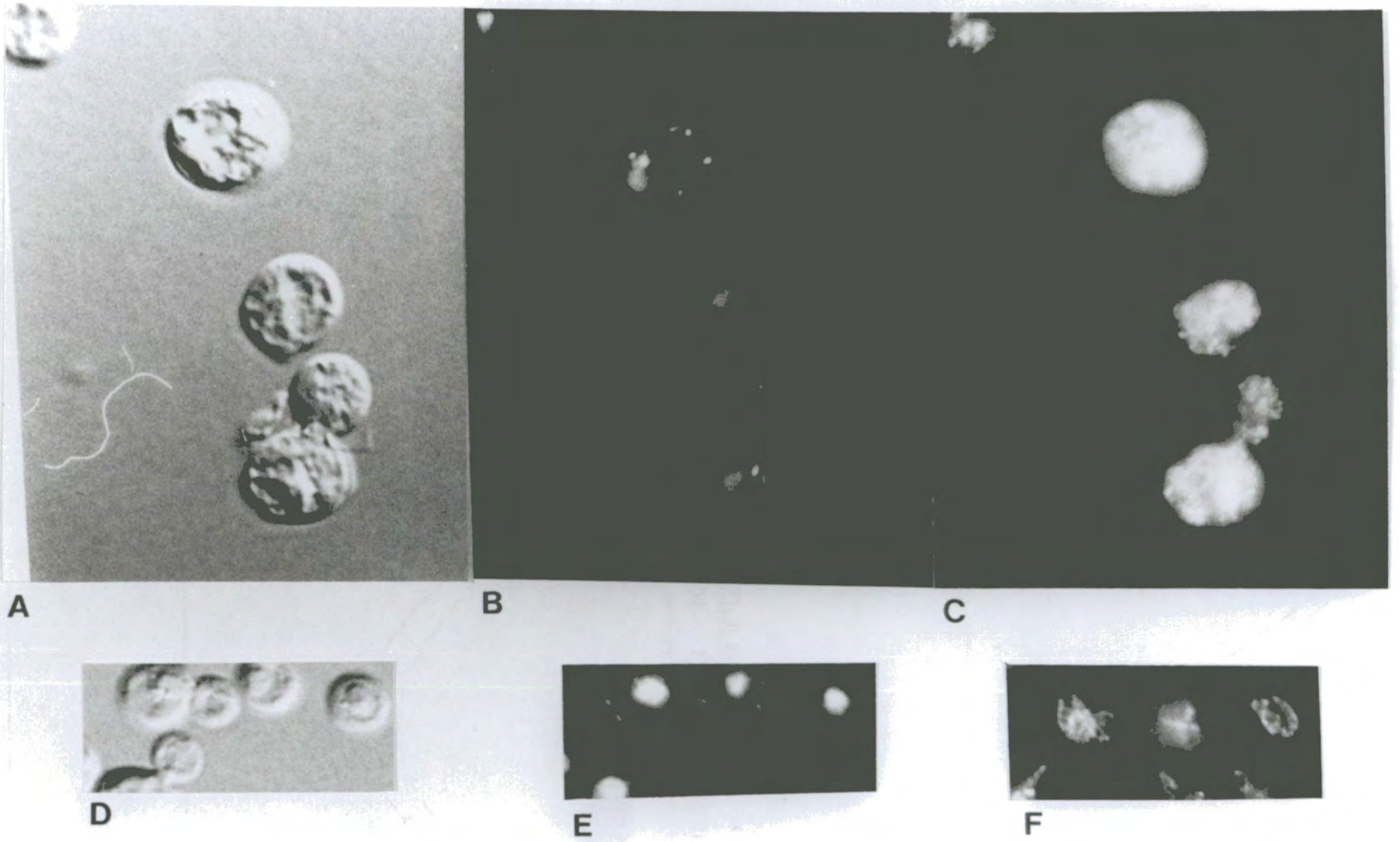
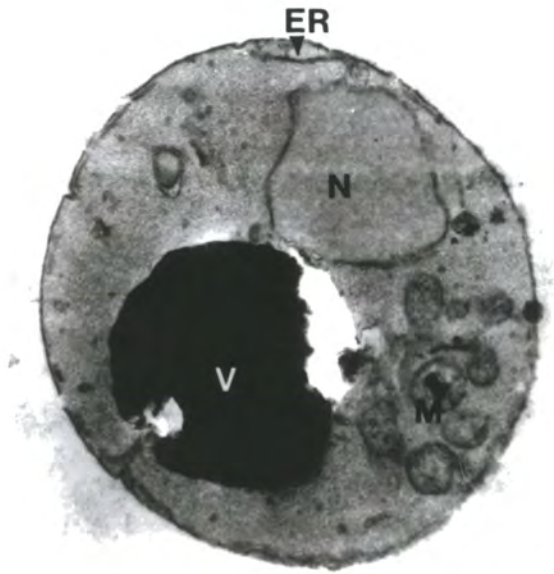
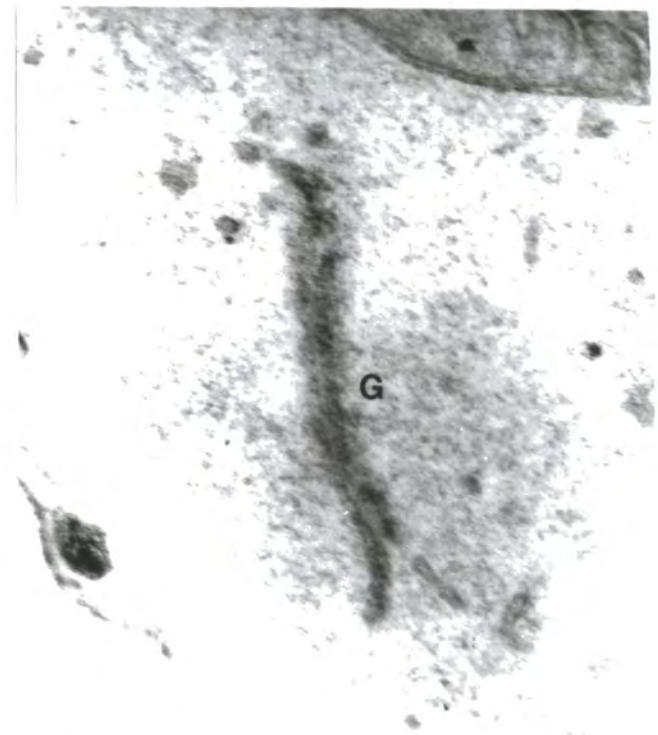

A
B
C
D
E
F

Figure 6.2: Electron microscopic examination of SEY6210 (wild type) (A) and MLY1511 (*ssv7*-1) (B and C) of ferrocyanide-reduced osmium - thiocarbohydrazide - ferrocyanide-reduced osmium fixation method. While this fixation method stains double membrane structures including the nucleus in the wild type (A), MLY1511 did not have such a nuclear membrane detectable in 45 sections examined (B). Instead, they have a number of Golgi like structures present in the cytoplasm (C). ER, endoplasmic reticulum; G, Golgi-like organelle; M, mitochondrion; N; nucleus; V, vacuole. Magnification: A, 19,500x; B, 17,000x; C, 60,000x.



A

B



C

To confirm that the putative anuclear phenotype of the *ssv7*-1 mutant was not a result of the fixation method used in preparing cells for immunofluorescence, wild type and mutant cells were examined by electron microscopy (Figure 6.2). In SEY6210 96.5% of 50 ultra-thinsections examined had a nucleus, and cells not exhibiting a nuclear structure were cut near the plasma membrane. MLY1511 cells did not have a single visible nucleus in 45 thin-sections examined, indicative of the absence of a nuclear membrane. Some Golgi-like organelles accumulated in some cells examined. Similar structures were also present in some other *ssv* mutants, such as *ssv9*-7 (Chapter 4). Alternatively, these structures could represent degenerate nuclear features. Mitochondria in *ssv7*-1 were proportional in size to the wild type, making it appear unlikely that the mutant affects mitochondrial morphogenesis to a visible extent.

6.2.2. *VPS* phenotype of *ssv7*-1

ssv7-1 secreted a small proportion of its p2CPY and properly localizes the remaining intracellular fraction to its 61 kDa mature form (Figure 6.3). This phenotype is indicative for a mutation in a gene not directly involved in protein sorting but other aspects, like vacuole biogenesis or nuclear morphogenesis. A number of other *ssv* mutants and also acidification defective mutants secrete a similar proportion of CPY. Alternatively, the mutation could affect a gene acting in both vacuole and nuclear morphogenesis.

6.2.3. Growth Behaviour of *ssv7*-1

It was expected that a mutant lacking a nuclear envelope would have a grossly altered growth pattern, because a number of regulatory processes rely on the selective import or exclusion of nuclear proteins in a cell cycle regulated fashion.

A comparison of the growth curves between *ssv7*-1 and its isogenic wild type shows an interesting correlation (Figure 6.4). Defining growth as the increase in cell number, the *ssv7*-1 mutant has a mean generation time of 3.5 hours compared to 1.75 hours for the wild type. By extrapolation from Figure 6.4., the mutant takes exactly

twice the time to bud and divide. Despite the differene in generation time, both cultures reach the stationary phase simultaneously after approximately 16 hours. Two explanations appear plausible. Firstly, *ssv7*-1 might be an unknown auxotrophy mutant whose auxotrophy requirement is depleted in the culture. The wild type not having this requirement would divide until some or all nutrients are depleted. Secondly, *ssv7*-1 could continue to grow as efficiently as the wild type in terms of increase of biomass, but division occurs half as frequently, leading to an increase in cell size. The latter possibility would also explain the increase in cell size observed with the *ssv7*-1 mutation. To test this hypothesis, the cell volume of log phase cultures of SEY6210 and MLY1511 in YPD was determined in a Coulter Counter (Figure 6.5). A comparison between the volume distribution of the wild type with the *ssv7*-1 mutant revealed that the *ssv7*-1 mutant is unable to control its cell volume (or cell size) resulting in cells of widely different volume. While the average unbuded wild type cell has a volume of 40 to 75 μm^3, the *ssv7*-1 cells have a volume from 20 to 500 μm^3. Light microscopic examination revealed that the budding was not affected in this mutant. Overall the rate of increase in volume of both mutant and wild type appear to be the same, and only the actual budding process or mitosis occurs more slowly.

6.2.4. Molecular Cloning and Characterization of the *SSV7* Locus

S. cerevisiae carrying *ssv* mutations are unable to grow on YPD medium supplemented with 1.5 M NaCl. This was exploited to develop a cloning strategy to clone the gene corresponding to the *ssv7*-1 mutation. MLY1511 (*ssv7*-1, *ura3*-52) cells were transformed with a YCp50 based yeast genomic DNA library (Chapter 5), and *URA3* transformants on yeast minimal medium selected for. A total of 3847 URA^+ transformants were obtained, of whih three conferred salt resistant growth. 3-Fluoroorotic acid curing of the plasmids was not successful, suggesting that the plasmids confer a selective advantage to the *ssv7*-1 mutants that make accidental loss or curing an unfavourable event.

Figure 6.3: Secretion of CPY in SEY6210 (wild type) and MLY1511 (*ssv7*-1). The immunoprecipitation of CPY from cells (P) and culture supernatant (S) are shown. The 68 kDa p2CPY precursor is secreted by the *ssv7*-1 mutant while the 61 kDa mature form is retained intracellularly. The wild type does not mislocalize any CPY extracellularly.

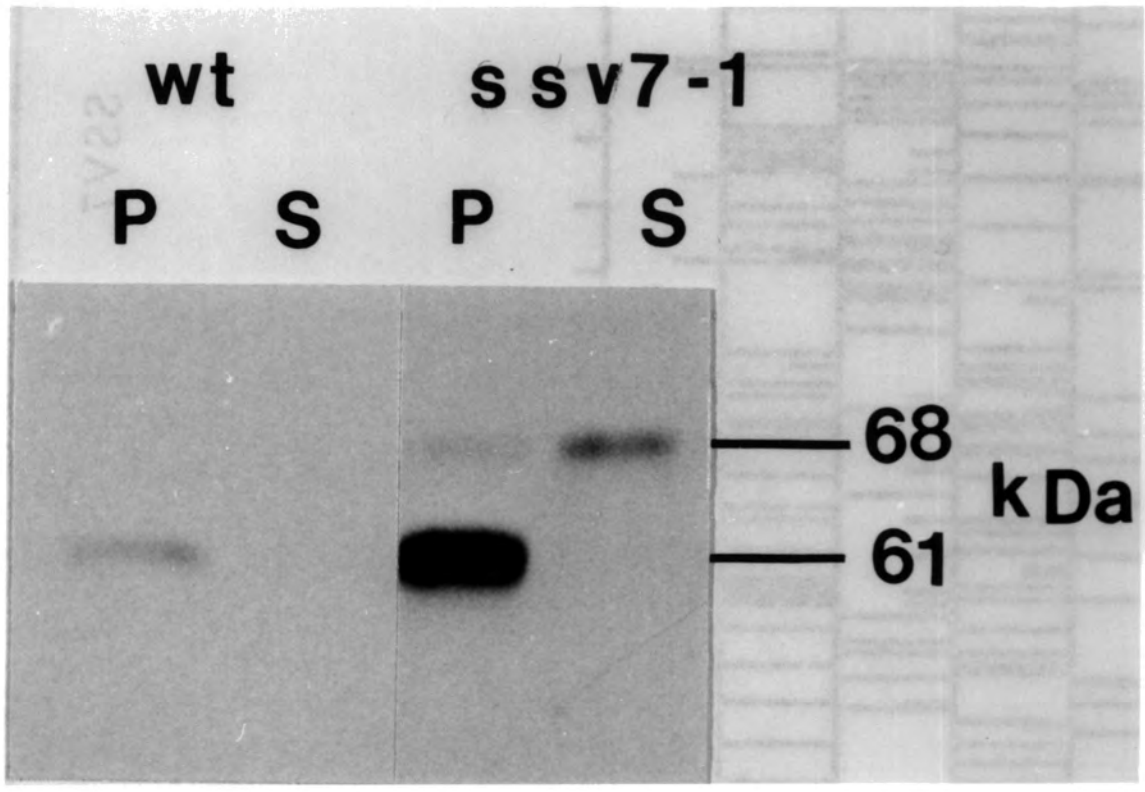
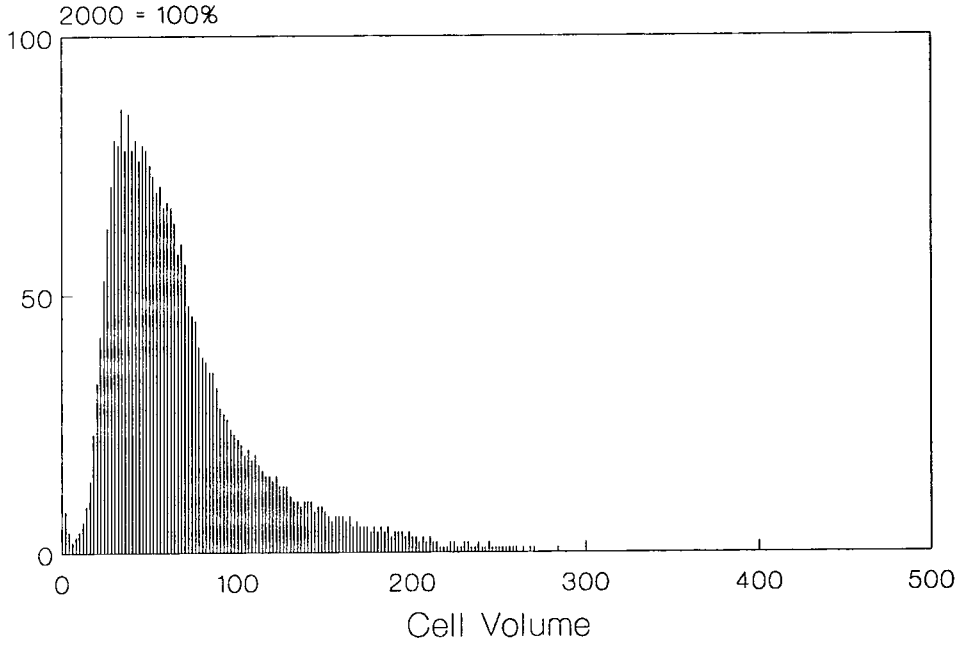
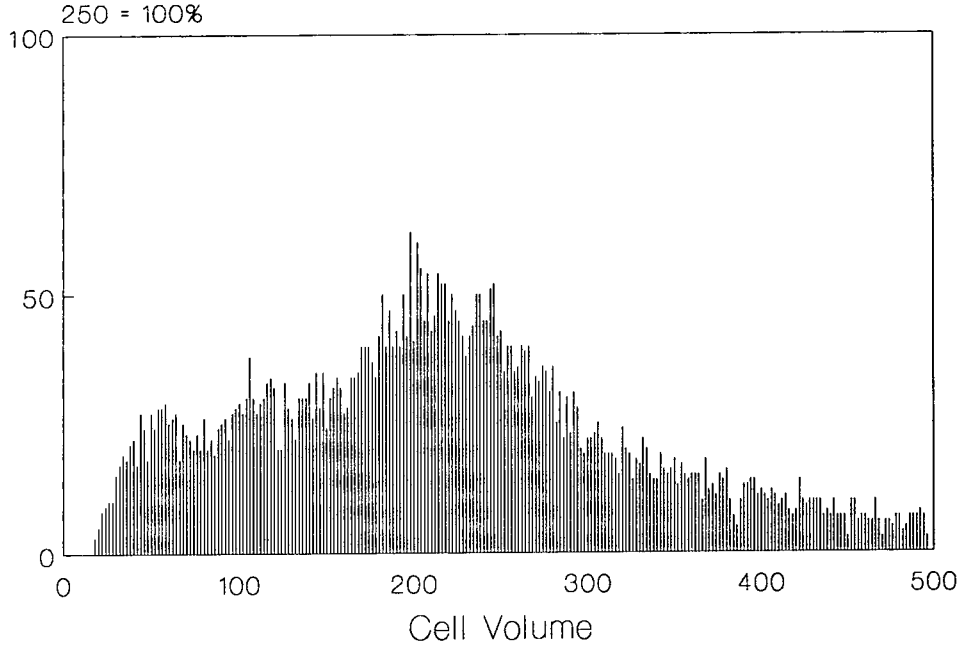

wt
ssv7-1
P
S
P
S
68
61
kDa

Figure 6.4: Growth curves of SEY6210 (wild type) and MLY1511 (*ssv7*-1) in YPD at 30°C and 140 rpm. 200 µL samples were taken in regular intervals, the total cell mumber was determined using a Coulter Counter and plotted against time. Each time point represents the average of three independent measurements. The mean generation time was determined by extrapolation from the graph during the cultures log phase.

wt

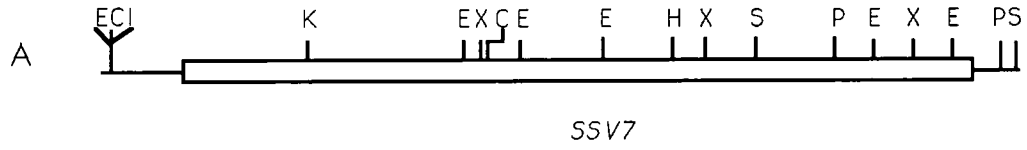

2000 = 100%
100
50
0
0
100
200
300
400
500
Cell Volume

ssv7-1


250 = 100%
100
50
0
0
100
200
300
400
500
Cell Volume

Plasmid DNA was isolated from each of the three yeast transformants, amplified in *E. coli* HB101, and reintroduced into MLY1511. All three plasmids, pMLY36, pMLY37 and pMLY38 complemented the salt sensitive phenotype associated with the *ssv* mutation. pMLY38 not only restored growth under osmotic stress but also enhanced growth in an allele specific manner in that the *ssv7*-1 mutant strain harbouring pMLY38 grew faster and to larger colonies on YPD + 1.5 M NaCl than the wild type on YPD only. This plasmid when transformed into SEY6210 did not lead to the same growth behaviour suggesting that pMLY38 is an allele specific low copy enhancer/suppressor. Preliminary restriction enzyme mapping demonstrated that pMLY36 and pMLY37 contained overlapping genomic DNA inserts, while pMLY38 had an unrelated restriction pattern (results not shown). Since both pMLY36 and pMLY37 restored wild type growth in the mutant and rescued the mutant phenotypes, it was assumed that both clones represent the genuine *SSV7* locus. Plasmid pMLY38 was not further analysd in this study. Because the 4.9 kb genomic DNA insert of pMLY36 was entirely contained within that of the 14 kb fragment of pMLY37, only pMLY36 was analysed further. pMLY36 was transformed into representative alleles of all other *ssv* complementation groups, but except for *ssv7*-1 it failed to restore osmoresistant growth in all other alleles tested. It also did not restore a wild type phenotype in a mutant carrying the *ssv7*-2 allele, indicating that either the mutant has a second osmosensitivity confering mutation whose defect is not rescued by pMLY36, or that its previous complementation pattern was caused by intergenic non-complementation associated with genetically interacting genes (the latter appears to be the case; M.D. Watson, personal communication). A detailed restriction analysis was carried out, digesting pMLY36 with all available enzymes alone or in combination (Figure 6.6 A). Smaller fragments of the genomic DNA fragment contained within pMLY36 were subcloned into pRS316, an *URA3* amp^r centromeric shuttle vector, and transformed into MLY1511. None of the smaller subclones was able to complement the *ssv7*-1 mutation, and it was concluded that most of the genomic DNA insert in pMLY36 represents the coding region of *SSV7* (Figure 6.6 B) and is necessary to restore gene function, unlike in some cases where partial subclones are able to complment a mutation (Erdmann *et al.*, 1991).

Figure 6.6: Restriction map and subcloning strategy to find the smallest possible complementing subclone. pMLY36 was restriction digested in single and double digests, and a restriction map was constructed (A). The assignment of restriction sites was unambiguous except for some *Eco*RI sites downstream of the *Sal*I site, because fragments were very similar in size. Fragments of the genomic clone were subcloned into pRS316 (B). *Sph*I cut fragments were blunt ended and ligated into the *Sma*I site. Constructs were transformed into MLY1511 (*ssv7*-1), and *URA3* transformants were tested for their osmosensitive phenotype. Subclones complemented the *ssv7*-1 mutation if they were able to sustain growth on YPD + 1.5 M NaCl. Restriction enzymes: C, *Cla*I; E, *Eco*RI; H, *Hpa*I; I, *Hind*III; P, *Sph*I; S, *Sal*I; X, *Xba*I.


A
E C I
K
E X C E
E
H X
S
P E X E
P S
SSV7

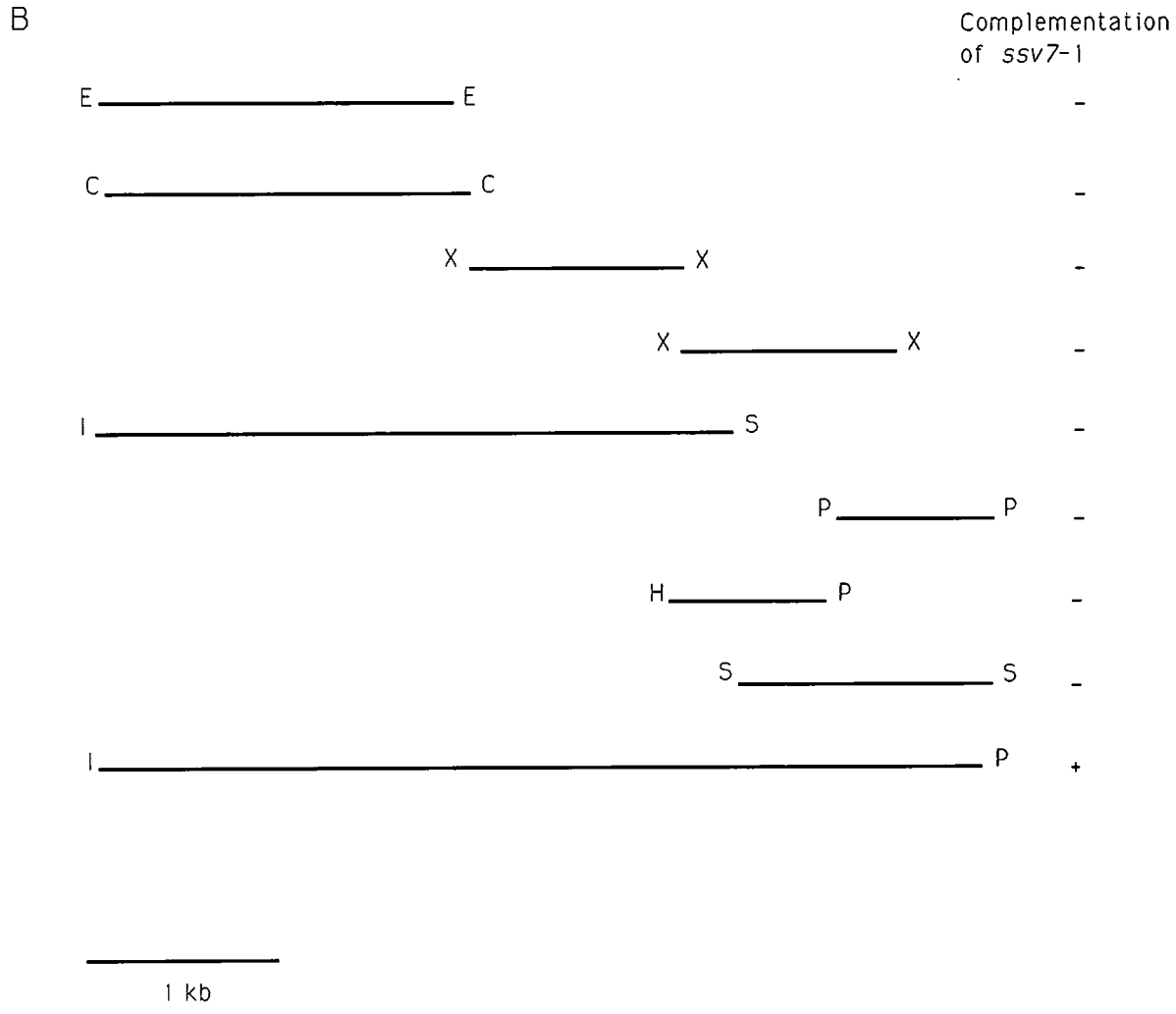

B
Complementation of ssv7-1
E
E
-
C
C
-
X
X
-
X
X
-
I
S
-
P
P
-
H
P
-
S
S
-
I
P
+
1 kb

Figure 6.7: Typical sequencing gel autoradiograph. The sequencing gel represents the genomic DNA sequence of SSV7 upstream of the *Hpa*I site. The autoradiograph was read twice manually and was assembled using the UWGCG package.

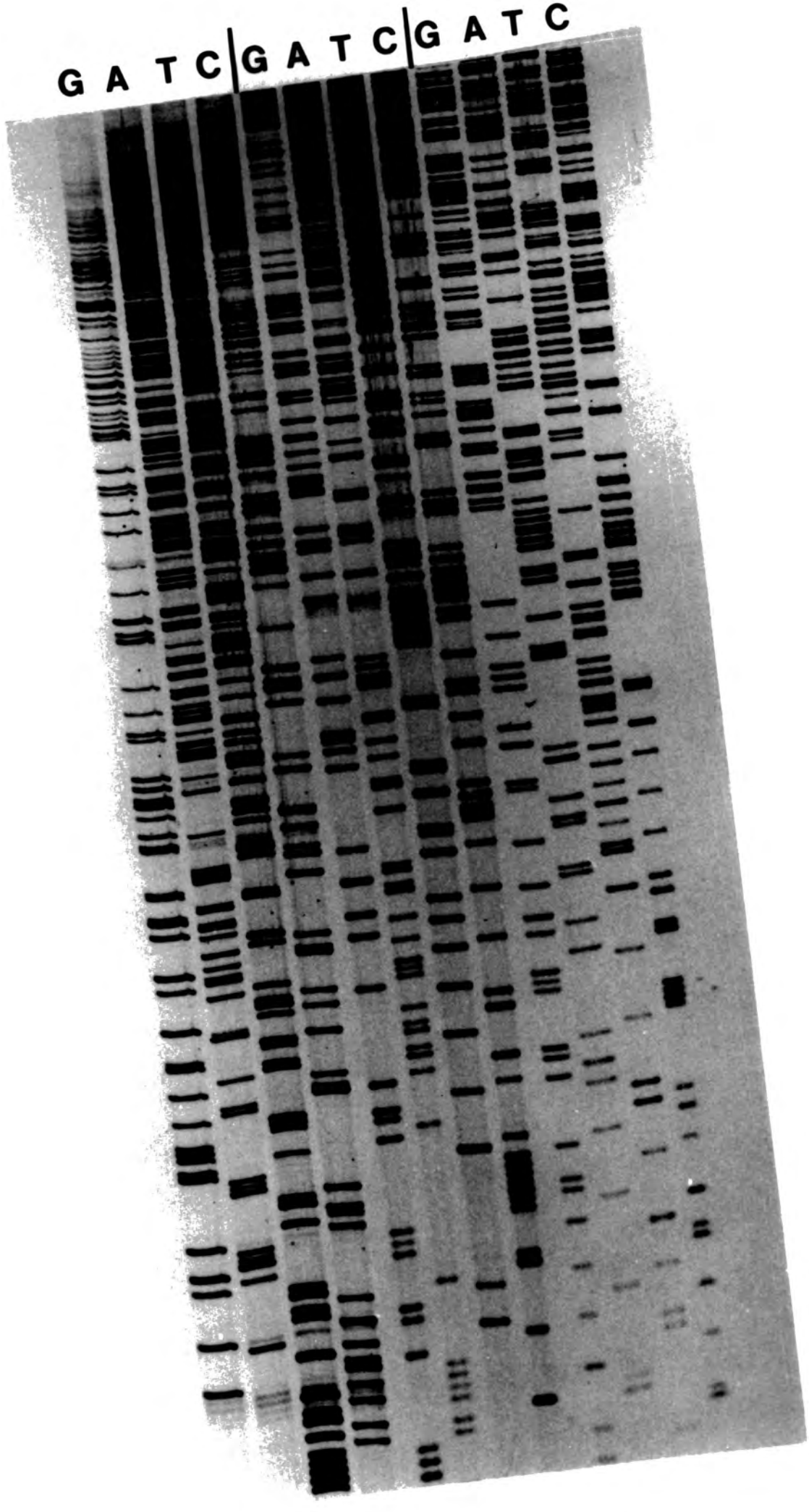

G A T C
G A T C
G A T C

Figure 6.8: Sequencing strategy of *SSV7*. Subclones in pKS$^-$ of the genomic clone containing the *SSV7* gene were sequenced using standard reverse and T7 sequencing primers. Some sequences were established using custom synthesized oligonucleotides as primers based on regions of known sequence. Custom made oligonucleotides are represented as black circles. Restriction enzymes: C, *Cla*I; E, *Eco*RI; H, *Hpa*I; I, *Hind*III; P, *Sph*I; S, *Sal*I; X, *Xba*I.

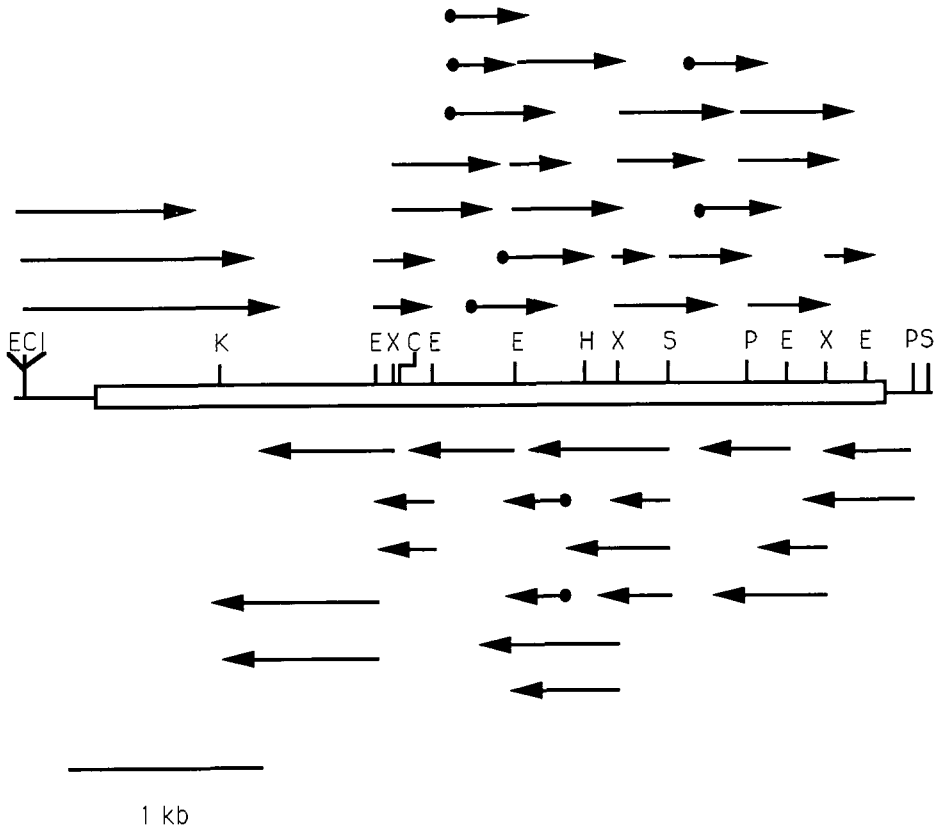
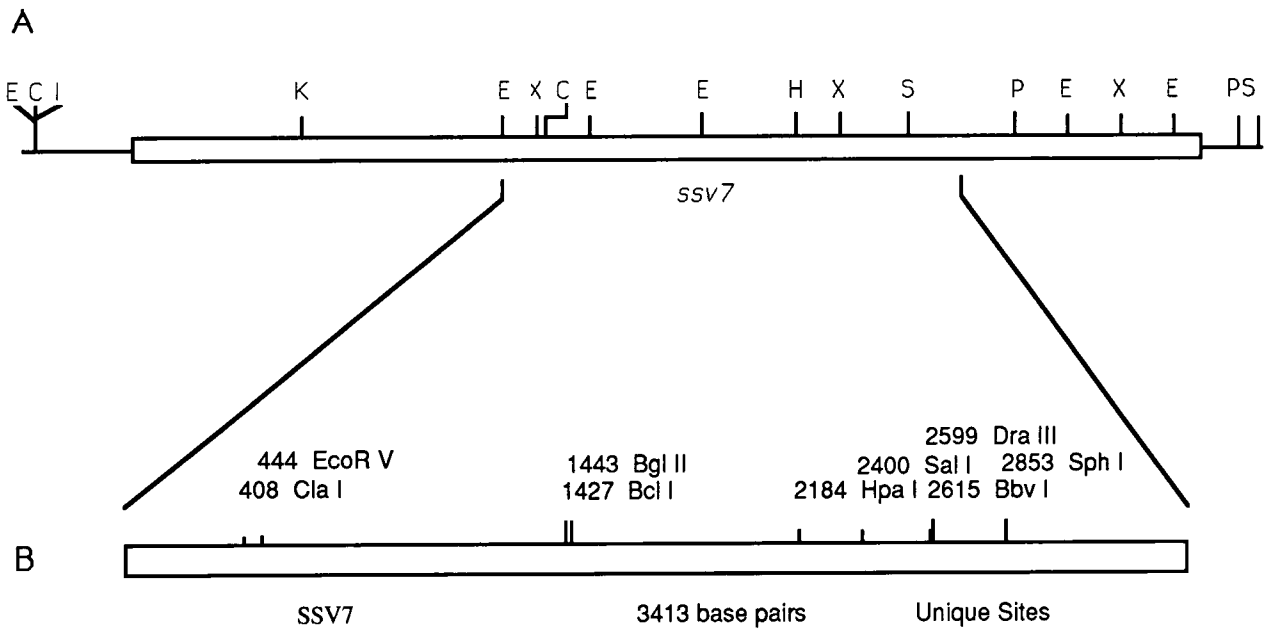

ECI
K
EXCE
E
H
X
S
P
E
X
E
PS
1 kb

Figure 6.9: Restriction map derived from the SSV7 sequence analysis and open reading frame map. A good correlation between the established and the deduced restriction map of SSV7 was observed (A), the only discrepancy being in the position of the *Sph*I site which is explainable by experimental error in determining the precise size of the restriction fragments. The subclone sequenced has one large open reading frame in the forward direction (B) and no significant other open reading frames. Short vertical lines in the open reading frame (ORF) map represent initiator codons (ATG), long vertical lines stop codons (TAA, TGA, TAG). Restriction enzymes: C, *Cla*I; E, *Eco*RI; H, *Hpa*I; I, *Hind*III; P, *Sph*I; S, *Sal*I; X, *Xba*I.


A
E C I
K
E X C E
E
H X
S
P E X E
PS
ssv7
2599 Dra III
444 EcoR V
1443 Bgl II
2400 Sal I
2853 Sph I
408 Cla I
1427 Bcl I
2184 Hpa I
2615 Bbv I
B
SSV7
3413 base pairs
Unique Sites

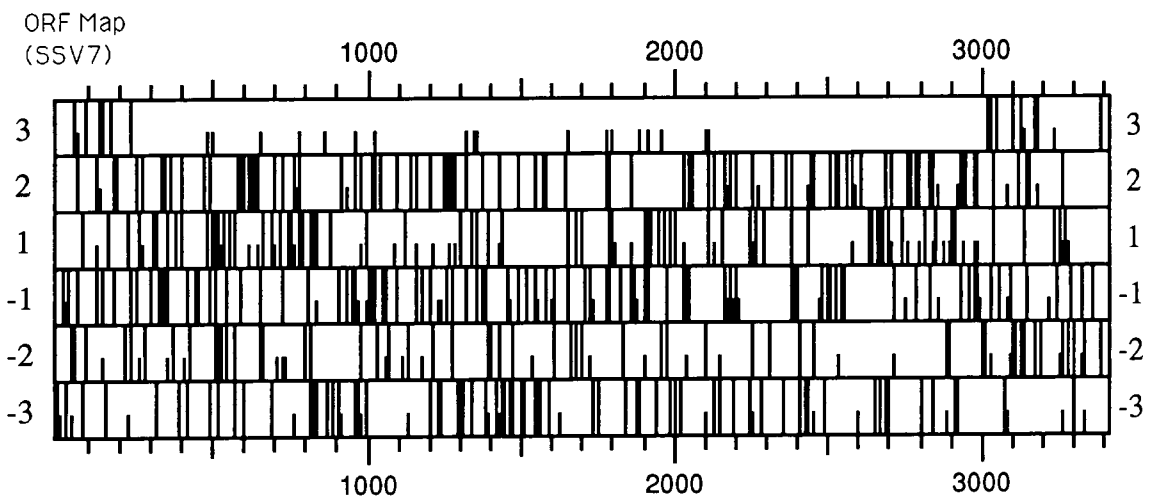

ORF Map
(SSV7)
1000
2000
3000
3
2
1
-1
-2
-3
1000
2000
3000

Figure 6.10: Genomic nucleotide sequence of *SSV7* and the predicted amino acid sequence. Putative TATA boxes upstream of the initiator codon are underlined in blue. Stop codons immediately adjacent to the open reading frame are underlined in red. A putative transcriptional terminator region is underlined in black.

```
   1 ATTTATTTGCCTTCCCACATTGTTTTACTTCATTTGCCTTCAATACCGTTCATTACGTTT  60

  61 AGCGAATGGCTAAGGCCCTTGTATTAGTAAAAGCCTAGAAAAAAAAAAAAAAAAAAAAA  120

 121 AAGGATATGATGTTATATGATACGTGTAGTTCATGTATTTATCTTCGGTAGTATAGGGCA  180

 181 GATTTAAGACTGAGTGTGCACGCTTCCAAAGTTTTTTTTACTATTTGATACATGCTTAAG  240

 241 TT  ATG GAG CAG AAT ATT ATT AGT ACC ATA AGG GAT GAG TGT ATT  284
   1     M   E   Q   N   I   I   S   T   I   R   D   E   C   I   14

 285 CGT CAC CGG TCG AAG TAC CTT ACG ATA GCA CAA CTA ACC GCT ATT  329
  15 R   H   R   S   K   Y   L   T   I   A   Q   L   T   A   I   29

 330 GCA GAG GCT AAA ATT AAC GAA TTC ATC ATA ACT GGT AAG GCA AAA  374
  30 A   E   A   K   I   N   E   F   I   I   T   G   K   A   K   44

 375 GAT CAA GAT TTG AGC AGT CTT CTA GAT AAA TGC ATC GAT ATT TTA  419
  45 D   Q   D   L   S   S   L   L   D   K   C   I   D   I   L   59

 420 TCT ATT TAC AAG AAG AAC TCG AAA GAT ATC AAA AAT ATT ATA TCG  464
  60 S   I   Y   K   K   N   S   K   D   I   K   N   I   I   S   74

 465 TGC AAA AAT AAG GGT GCA ATG ATT AGT TCA AAT TCC GTA ATG ATT  509
  75 C   K   N   K   G   A   M   I   S   S   N   S   V   M   I   89

 510 ATT CAA TTA AAT TAT GTT TAC TAC AAG GTA ATT CAC ATT ATT GTA  554
  90 I   Q   L   N   Y   V   Y   Y   K   V   I   H   I   I   V  104

 555 ACA ACC AAT ATT CCT CAT TTA AGT GAA TTC GCC AAG ATT AAA TTA  599
 105 T   T   N   I   P   H   L   S   E   F   A   K   I   K   L  119

 600 CAT AAG AGC ACG AGT GAT GAG GGC AAC GGT AAT AAC AAC AAT AAT  644
 120 H   K   S   T   S   D   E   G   N   G   N   N   N   N   N  134

 645 GAA TTT CAA CTC ATG AAC ATT TAC AAC ACT TTG CTG GAA ACC TTA  689
 135 E   F   Q   L   M   N   I   Y   N   T   L   L   E   T   L  149

 690 TTA AAA GAT GAA AAC ATT GCA AAA ATA AAA AGT TTC ATT AAG TCT  734
 150 L   K   D   E   N   I   A   K   I   K   S   F   I   K   S  164

 735 TCC ATA AAA CAA ACA AAA TTG AAC CAT GAG CAA GAA GAA TGT AAC  779
 165 S   I   K   Q   T   K   L   N   H   E   Q   E   E   C   N  179

 780 CTG ATG AGA ACG GGT TCC TAT ATC ACT TCC AAT CAA TTA AAC TCC  824
 180 L   M   R   T   G   S   Y   I   T   S   N   Q   L   N   S  194

 825 CTA ATA AGT TCA TCA GCA AAT TCT GCT TCC TCC CAA ATG GAG ATA  869
 195 L   I   S   S   S   A   N   S   A   S   S   Q   M   E   I  209

 870 CTA CTG ATA GAT ATA CGA TCA AGG TTG GAA TTC AAC AAG TCA CAT  914
 210 L   L   I   D   I   R   S   R   L   E   F   N   K   S   H  224

 915 ATT GAT ACA AAA AAT ATT ATA TGC CTG GAG CCT ATT TCT TTT AAA  959
 225 I   D   T   K   N   I   I   C   L   E   P   I   S   F   K  239

 960 ATG TCA TAT TCA GAT CAT GAT TTG GAG AAA AAA TCA TTA ATT ACT 1004
 240 M   S   Y   S   D   H   D   L   E   K   K   S   L   I   T  254

1005 TCT CCT AAT AGT GAG ATT AAA ATG TTT CAA AGT AGA AAT CTT TTC 1049
 255 S   P   N   S   E   I   K   M   F   Q   S   R   N   L   F  269

1050 AAG TTT ATC ATT CTC TAT ACA GAC GCA AAC GAA TAC AAT GTT AAA 1094
 270 K   F   I   I   L   Y   T   D   A   N   E   Y   N   V   K  284

1095 CAG CAG TCT GTC CTG TTG GAC ATT CTG GTG AAT CAT TCC TTT GAA 1139
 285 Q   Q   S   V   L   L   D   I   L   V   N   H   S   F   E  299

1140 AAA CCA ATA TCC GAT GAC TTT ACC AAA ATT TTC ATT CTG GAA TCT 1184
 300 K   P   I   S   D   D   F   T   K   I   F   I   L   E   S  314

1185 GGT TTT CCA GGT TGG CTT AAG TCA AAT TAT GGG AGG AAA GTA TCA 1229
 315 G   F   P   G   W   L   K   S   N   Y   G   R   K   V   S  329

1230 TCA TCT TTT CCA TCA AAT AAC AAT ATT AAG GAT GAT AGT GTT TAT 1274
 330 S   S   F   P   S   N   N   N   I   K   D   D   S   V   Y  344
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1275 TTT AAT GGT AAC ACT TCT GGC CTA AGT TTA CAA CAT TTA CCT AAG 1319
 345 F   N   G   N   T   S   G   L   S   L   Q   H   L   P   K   359

1320 ATG TCT CCC AGT ATA AGA CAT TCA ATG GAC GAC TCT ATG AAA GAA 1364
 360 M   S   P   S   I   R   H   S   M   D   D   S   M   K   E   374

1365 ACT GCT AGT TGG CCT ACT CCA TTA ATT CAT CTT CAA CAA CAG CAA 1409
 375 T   A   S   W   P   T   P   L   I   H   L   Q   Q   Q   Q   389

1410 CAA CAG CAA TCA GAC AAT GAT CAT GTG CTA AAA AGA TCT TCA AGT 1454
 390 Q   Q   Q   S   D   N   D   H   V   L   K   R   S   S   S   404

1455 TTC AAA ACA TTA TTC TCA AAT TAT ACG TCT CCT AAT CCG AAG AAT 1499
 405 F   K   T   L   F   S   N   Y   T   S   P   N   P   K   N   419

1500 TCA AAT TCA AAC TTA TAT TCT ATA TCT TCG TTG TCC ATA TCT AGT 1544
 420 S   N   S   N   L   Y   S   I   S   S   L   S   I   S   S   434

1545 TCA CCA TCG CCT TTA CCT CTA CAT TCG CCT GAC CCA GTT AAG GGC 1589
 435 S   P   S   P   L   P   L   H   S   P   D   P   V   K   G   449

1590 AAT TCA TTG CCA ATC AAT TAT CCG GAA ACG CCA CAT CTT TGG AAA 1634
 450 N   S   L   P   I   N   Y   P   E   T   P   H   L   W   K   464

1635 AAC AGT GAG ACA GAT TTT ATG ACA AAT CAA AGA GAA CAG TTG AAT 1679
 465 N   S   E   T   D   F   M   T   N   Q   R   E   Q   L   N   479

1680 CAC AAC TCT TTT GCT CAC ATA GCT CCT ATC AAC ACG AAG GCC ATC 1724
 480 H   N   S   F   A   H   I   A   P   I   N   T   K   A   I   494

1725 ACT TCT CCA TCA AGA ACT GCC ACA CCG AAG TTA CAA CGC TTC CCG 1769
 495 T   S   P   S   R   T   A   T   P   K   L   Q   R   F   P   509

1770 CAA ACA ATT AGT ATG AAC CTT AAT ATG AAC TCC AAT GGA CAC AGT 1814
 510 Q   T   I   S   M   N   L   N   M   N   S   N   G   H   S   524

1815 TCT GCC ACC TCT ACC ATT CAA CCT TCG TGT CTA TCC TTG TCT AAT 1859
 525 S   A   T   S   T   I   Q   P   S   C   L   S   L   S   N   539

1860 AAT GAC TCT TTC AGA TCA TAC GAG ATG TTA CAC CAA CTT CTT CTC 1904
 540 N   D   S   F   R   S   Y   E   M   L   H   Q   L   L   L   554

1905 ATA ATT ATG ACC TTG ATT TCG CGG TTG GTT TGG GAA AAC CTA GAA 1949
 555 I   I   M   T   L   I   S   R   L   V   W   E   N   L   E   569

1950 ATC CCG TGT TAC ATG AAC TGT ATC ATT CAG TGT ACC TTA GGT ACA 1994
 570 I   P   C   Y   M   N   C   I   I   Q   C   T   L   G   T   584

1995 CAC GAA TTA ACC CAA ATC TTT TTG GAC GAT TCA TAT GCT AAA CAC 2039
 585 H   E   L   T   Q   I   F   L   D   D   S   Y   A   K   H   599

2040 ATC AAT ATT AAT AGT AAG TTG GGA TCG AAA GGT ATT CTG GCA AAA 2084
 600 I   N   I   N   S   K   L   G   S   K   G   I   L   A   K   614

2085 TAT TTT GCA AGG TTG GTT CAT ATG ATG TAT AAG GAA CAG GTT GAT 2129
 615 Y   F   A   R   L   V   H   M   M   Y   K   E   Q   V   D   629

2130 GGT TCA AAG AAA ATT TCC ATA TCA CCG ATA AAA TTT AAA TTG GCA 2174
 630 G   S   K   K   I   S   I   S   P   I   K   F   K   L   A   644

2175 TGT GGA TCT GTT AAC TCA TTA TTT AAG ACT GCA TCC CAA CAG GAC 2219
 645 C   G   S   V   N   S   L   F   K   T   A   S   Q   Q   D   659

2220 TGC CAA GAG TTT TGC CAA TTC CTT CTA GAT GGT CTT CAT GAA GAC 2264
 660 C   Q   E   F   C   Q   F   L   L   D   G   L   H   E   D   674

2265 TTG AAC CAA TGC GGT TCA AAC CCA CCT TTG AAG GAG CTT TCT CAA 2309
 675 L   N   Q   C   G   S   N   P   P   L   K   E   L   S   Q   689

2310 GAA GCT GAG GCG AGA AGA GAA AAA CTG TCT TTG CGA ATT GCC TCG 2354
 690 E   A   E   A   R   R   E   K   L   S   L   R   I   A   S   704

2355 TCA ATT GAG TGG GAA CGA TTC TTG ACT ACT GAT TTC AGT GTT ATT 2399
 705 S   I   E   W   E   R   F   L   T   T   D   F   S   V   I   719
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```
2400 GTC GAC TTA TTT CAG GGA CAA TAC GCC TCA CGA CTA AAA TGT AAA 2444
 720 V   D   L   F   Q   G   Q   Y   A   S   R   L   K   C   K   734

2445 GTC TGT AGT CAT ACC TCG ACA ACA TAC CAA CCT TTT ACG GTG CTG 2489
 735 V   C   S   H   T   S   T   T   Y   Q   P   F   T   V   L   749

2490 TCA ATC CCT ATT CCT AAA AAA AAT TCC CGA AAT AAT ATT ACC ATT 2534
 750 S   I   P   I   P   K   K   N   S   R   N   N   I   T   I   764

2535 GAA GAT TGT TTC AGA GAG TTC ACC AAA TGT GAG AAC TTG GAA GTG 2579
 765 E   D   C   F   R   E   F   T   K   C   E   N   L   E   V   779

2580 GAT GAG CAA TGG TTG TGC CCA CAT TGT GAA AAA AGG CAG CCC TCC 2624
 780 D   E   Q   W   L   C   P   H   C   E   K   R   Q   P   S   794

2625 ACG AAA CAA TTG ACA ATA ACG AGA TTA CCG AGG AAT CTG ATA GTC 2669
 795 T   K   Q   L   T   I   T   R   L   P   R   N   L   I   V   809

2670 CAT TTA AAG AGA TTT GAT AAT TTA TTA AAC AAA AAT AAT GAC TTC 2714
 810 H   L   K   R   F   D   N   L   L   N   K   N   N   D   F   824

2715 GTC ATA TAC CCT TTT TTG TTG GAC TTG ACT CCA TTT GGG GCC AAT 2759
 825 V   I   Y   P   F   L   L   D   L   T   P   F   G   A   N   839

2760 GAT TTT GAC GGG GTT TTT CCT CCA GGT GTT AAT GAC GAT GAA CTA 2804
 840 D   F   D   G   V   F   P   P   G   V   N   D   D   E   L   854

2805 CCA ATA AGG GGA CAA ATA CCA CCT TTT AAG TAT GAA TTA TAT GGT 2849
 855 P   I   R   G   Q   I   P   P   F   K   Y   E   L   Y   G   869

2850 GTA GCA TGC CAC TTT GGT ACT TTG TAT GGT GGT CAT TAT ACA GCC 2894
 870 V   A   C   H   F   G   T   L   Y   G   G   H   Y   T   A   884

2895 TAT GTG AAA AAG GGA TTA AAG AAG GGA TGG CTA TAT TTT AAT GAT 2939
 885 Y   V   K   K   G   L   K   K   G   W   L   Y   F   N   D   899

2940 ACC AAA TAT AAA CCT GTC AAA AAC AAA GCC GAT GCA ATT AAC TCT 2984
 900 T   K   Y   K   P   V   K   N   K   A   D   A   I   N   S   914

2985 AAT GCA TAC GTT TTG TTT TAT CAC CGC GTC TAC GGT GTT TGA TTCA 3030
 915 N   A   Y   V   L   F   Y   H   R   V   Y   G   V   •       928

3031 TTTGAATAAATAACTGAAAACCGTTGTCTATACACTTTTTTTCCCGTTCAACATGGCATA 3090

3091 TCATTATTTCCATCTAAGGTCAAAGTAAAAAACCCTAAATACAAATGATTTATTTTAACG 3150

3151 GTAACAAACGAACTTTTTTTTTAGAAGGTGTAAAGATGAGATCACGCCCAGATTTTGTCTT 3210

3211 CCTCCTGTGCATCTCTTGGGTTGATGATTTTCCTTTCTACCATAATACGTCATGAAAAGT 3270

3271 ATGTAAATATTTATGGGCTTACATATTATCTTTTATTTGGAGAAAATTCCCTCATTCCCA 3330

3331 GTCATCATCGTCGGTCGGTCGTCCTTGCTCTCACCCTTGTTGGGGGGGGCCACCTGGGGC 3390

3391 ACTAACCTTTTTCCAATCCCCCC                                       3413
```

Further downstream the nucleotide sequence ^{3270}TATG.....^{3282}TATG.....^{3301}TTTTA conformed the consensus of a transcriptional terminator (Zaret and Sherman, 1982). The immediate nucleotides preceding the ATG start codon are GTT which is similar to that found in a number of regulatory genes in the same position. Complete consensus sequences required for intron splicing in *S. cerevisiae* (Langford *et al.*, 1984) were not detected. Thus, *SSV7* appears not to contain introns. A hydrophobicity analysis of the deduced protein sequence (Kyte and Doolittle, 1982) indicates that Ssv7p is relatively hydrophilic and seems to have no N-terminal signal sequence and no outstanding uninterrupted stretches of α helix of β sheet (Figure 6.11 and 6.12). Therefore, this protein would not be expected to enter the secretory pathway by translocation into the ER lumen, or represent an intermediate filament-like protein characterized by a long uninterrupted α helical stretch. Some residues of the protein are acidic (Figure 6.11) which results in the protein having a predicted isoelectric point of 8.50 (Figure 6.13).

A protein sequence comparison of the deduced amino acid sequence with a consensus sequence library available on the UWGCG package revealed that the protein has seven putative N-linked glycosylation sites and a link-patch consensus from amino acid 792 to 807 (QPSTKQLTITRLPRNL) often found associated with proteins having separate functional domains. A number of other secondary consensus sequences were detected, the most prominent that for phosphorylation (Table 6.1). It has not yet been examined whether the protein is phosphorylated *in vivo*.

A comparison of the predicted amino acid sequence to other known protein sequences contained in all available protein sequence databases including a daily updated EMBL data base showed that the gene and protein have not been sequenced before and do not have any overall homology to other genes and proteins. Using the FASTA and TFASTA algorithms (Pearson and Lipman, 1988) it was found that the C-terminal 300 amino acids of Ssv7p were 20% identical and 42% homologous to the catalytic domains of nucleotide exchange factors such as the *S. cerevisiae* Cdc25p (Camonis *et al.*, 1986) and 16% identical and 41% homologous to Bud5p (Chant *et al.*, 1991; Powers *et al.*, 1991), another nucleotide exchange factor.

Figure 6.11: Secondary protein structure analysis of Ssv7p. The protein has no significant hydrophobic signal peptide required for translocation into the ER, nor does it have any distinct secondary structure defined by α helices or β sheets. A map representing acidic and basic residues reveals that the protein has a significant number of acidic and basic residues, of which the acidic residues are located near the C-terminus. The length of the vertical bars are proportional to the dissociation potential of acidic and basic residues. The Cys and His map suggests that the protein possibly has some tertiary structure not only defined by its primary amino acid sequence because some cysteines have a high potential to form cystines (long vertical bars) and the protein has a number of histidine residues (intermediate vertical bars) and other cysteines unlikely to undergo disulphide bridge formation (short vertical bars).

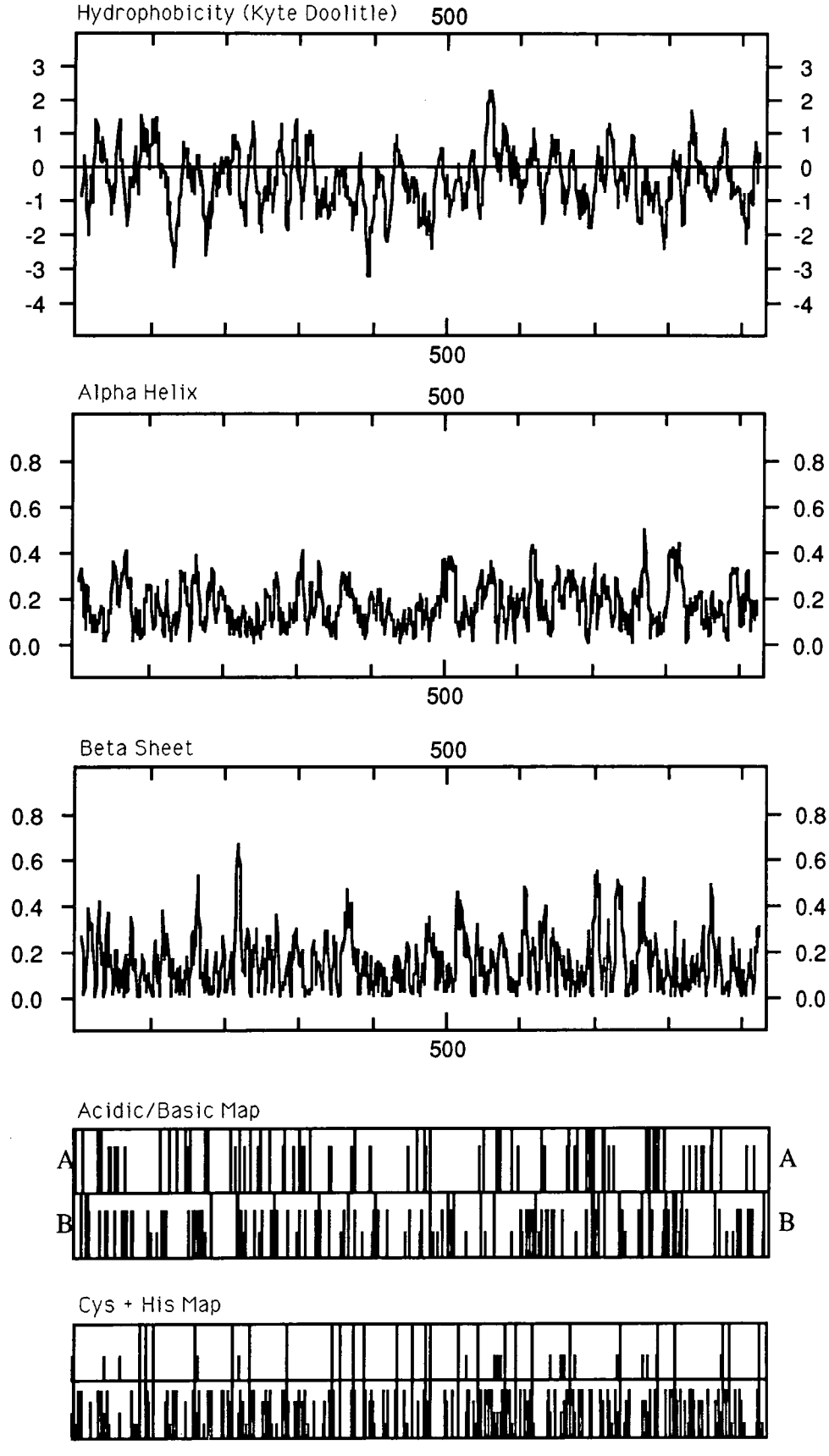

Hydrophobicity (Kyte Doolitle)
500
3
2
1
0
-1
-2
-3
-4
500
Alpha Helix
500
0.8
0.6
0.4
0.2
0.0
500
Beta Sheet
500
0.8
0.6
0.4
0.2
0.0
500
Acidic/Basic Map
A
B
Cys + His Map

Figure 6.12: Chou-Fasman prediction of the secondary structure of Ssv7p. The protein lacks any significant long α-helical structures and probably is not a cytoskeletal or structural element. Most short α-helical stretches coincide with charged residues which could function in recognition.

PLOTSTRUCTURE of: ssv7p.gcg ck: 5267

P1; Ssv7p - Saccharomyces cerevisiae -

Chou-Fasman Prediction
June 20, 1992 20:59

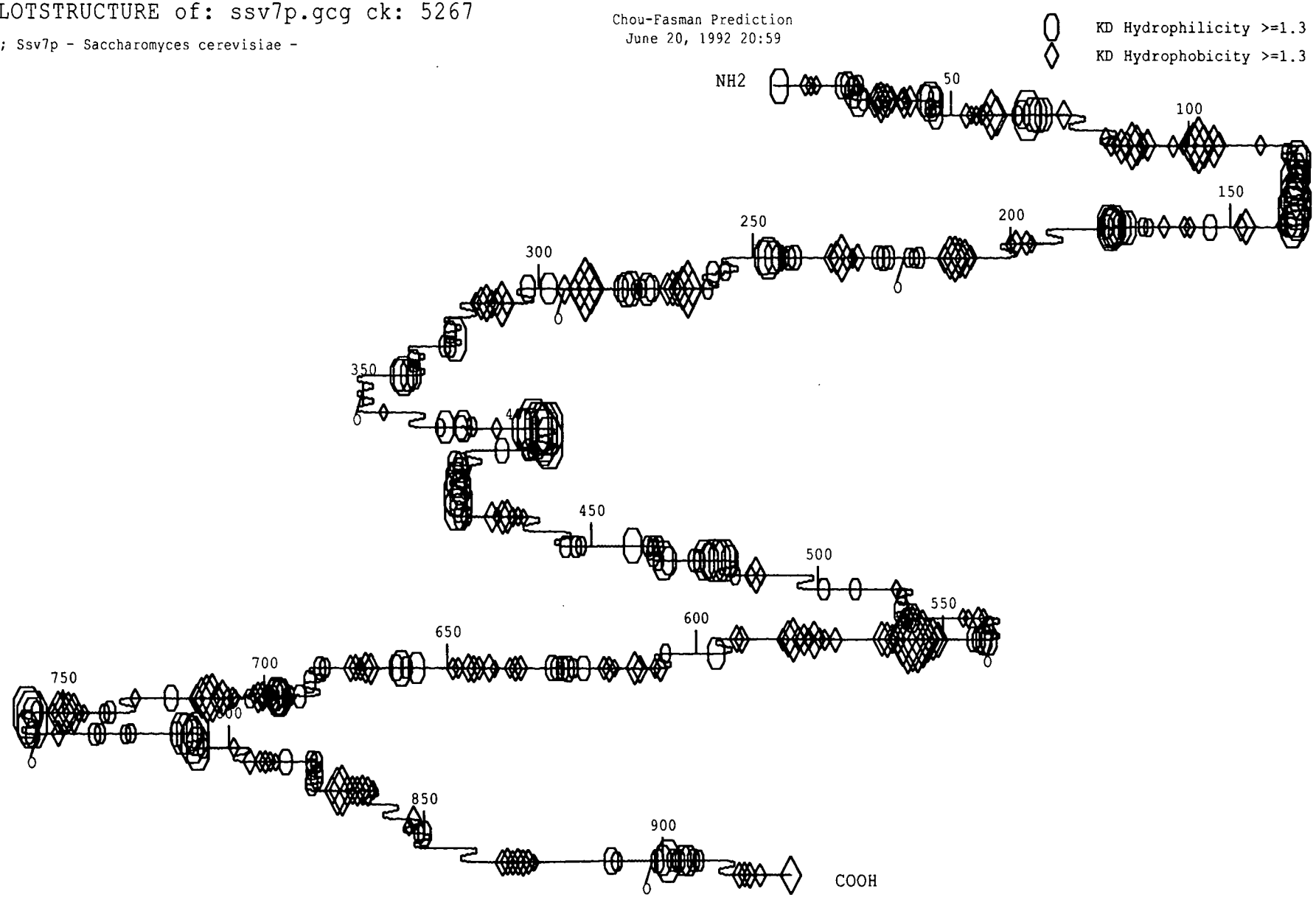
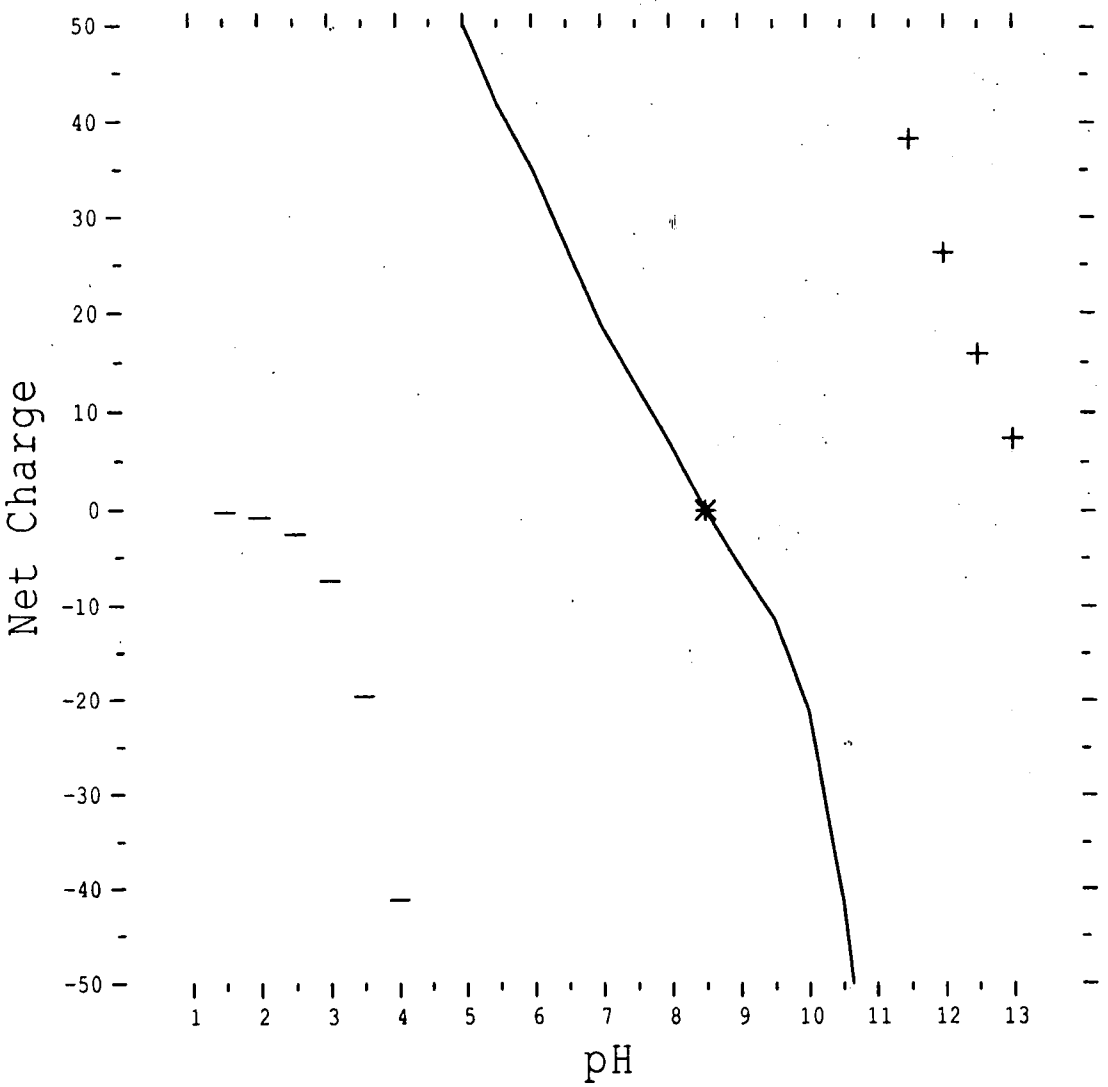


Figure 6.13: Isoelectric point map of Ssv7p. The isoelectric point of Ssv7p was calculated with the help of the UWGCG package. An isoelectric point of 8.5 indicates that the protein's net charge is 0 at a pH 8.5, typical for basic proteins.

ISOELECTRIC of: Ssv7p.Gcg Ck: 5267 1 to 927 May 3, 1992 12:11

* = Isoelectric point = 8.50



Charged Groups

Arg	29
Lys	73
His	28
Tyr	33
Cys	20
Glu	45
Asp	44

Conensus sites	Sequence		
Amidation	xG(R,K)(R,K) xG(R)(K)		
	324: WLKSN	**YGRK**	VSSSF
Asn-Glycosylation	N~(P)(S,T)~(P) N~P(S)~P		
	221: SRLEF	**NKSH**	IDTKN
	295: LDILV	**NHSF**	EKPIS
	348: VYFNG	**NTSG**	LSLQH
	411: KTLFS	**NYTS**	PNPKN
	540: LSLSN	**NDSF**	RSYEM
	761: KNSRN	**NITI**	EDCFR
	898: GWLYF	**NDTK**	YKPVK
cAMP-phosphorylation site	(R,K)2x(S,T) (K){2}x(S)		
	63: ILSIY	**KKNS**	KDIKN
	326: KSNYG	**RKVS**	SSFPS
	400: NDHVL	**KRSS**	SFKTL
	632: QVDGS	**KKIS**	ISPIK
	755: SIPIP	**KKNS**	RNNIT
CK2-phosphorylation site	(S,T)x2(D,E) (T)x{2}(D)		
	8: QNIIS	**TIRD**	ECIRH
	50: DQDLS	**SLLD**	KCIDI
	122: IKLHK	**STSD**	EGNGN
	123: KLHKS	**TSDE**	GNGNN
	144: MNIYN	**TLLE**	TLLKD
	205: ANSAS	**SQME**	ILLID
	216: LIDIR	**SRLE**	FNKSH
	223: LEFNK	**SHID**	TKNII
	241: ISFKM	**SYSD**	HDLEK
	243: FKMSY	**SDHD**	LEKKS
	367: PSIRH	**SMDD**	SMKET
	371: HSMDD	**SMKE**	TASWP
	393: QQQQQ	**SDND**	HVLKR
	466: HLWKN	**SETD**	FMTNQ
	538: SCLSL	**SNND**	SFRSY
	656: LFKTA	**SQQD**	CQEFC
	704: SLRIA	**SSIE**	WERFL
	763: SRNNI	**TIED**	CFREF
	772: CFREF	**TKCE**	NLEVD
Myristyl	G~(E,D,K,R,H,P,Y,F,W)x2(S,T,A,G,C,N)~(P) G~(E,D,K,R,H,P,Y,F,W)x{2}(S)~P		
	79: SCKNK	**GAMISS**	NSVMI
	127: STSDE	**GNGNNN**	NNEFQ
	129: SDEGN	**GNNNNN**	EFQLM
	184: NLMRT	**GSYITS**	NQLNS
	347: SVYFN	**GNTSGL**	SLQHL
	646: FKLAC	**GSVNSL**	FKTAS
	725: VDLFQ	**GQYASR**	LKCKV
	875: VACHF	**GTLYGG**	HYTAY
	879: FGTLY	**GGHYTA**	YVKKG
	889: AYVKK	**GLKKGW**	LYFND

Table 6.1 - continued on next page.

Conensus sites		Sequence	
PKC-phosphorylation site		(S,T)x(R,K) (T)x(R)	
	8: QNIIS	**TIR**	DECIR
	40: NEFII	**TGK**	AKDQD
	74: IKNII	**SCK**	NKGAM
	165: SFIKS	**SIK**	QTKLN
	237: CLEPI	**SFK**	MSYSD
	363: PKMSP	**SIR**	HSMDD
	371: HSMDD	**SMK**	ETASW
	404: LKRSS	**SFK**	TLFSN
	502: PSRTA	**TPK**	LQRFP
	542: LSNND	**SFR**	SYEML
	631: EQVDG	**SKK**	ISISP
	699: RREKL	**SLR**	IASSI
	794: EKRQP	**STK**	QLTIT
Tyr-phosphorylation site		(R,K)x{2,3}(D,E)x{2,3}Y (K)x{2}(D)x{3}Y	
	820: DNLLN	**KNNDFVIY**	PFLLD

Table 6.1: Consensus sites of the predicted *SSV7* protein. The deduced protein sequence of Ssv7p was searched with a consensus library using the MOTIF search on the UWGCG package. Amino acids of Ssv7p perfectly matching with the putative consensus sequences (displayed above alignment) are highlighted in boldface, and their location in the Ssv7 protein is indicated.

Since the homology was conserved over a region which has been associated with nucleotide exchange function, and the conservation between different exchange factors usually never exceeded 20% identity and 45% similarity within one species (Chant *et al.*, 1991), there is good reason to propose that based on sequence analysis Ssv7p has a consensus for mediating a nucleotide exchange process (Figure 6.15). The N-terminal 400 amino acids has homology to Gramicidin S synthetase I (GrsA) from *Bacillus brevis* (Krätzschmar *et al.*, 1989). The N-terminal domain of GrsA was homologous to the N-terminus of tyrocidine synthetase, both of which are involved in thioester linkage formation between phenylalanine and subsequent amino acids of the peptide antibiotic. The reaction is characterized by the initial binding of phenylalanine to a conserved cysteine. However, the particular conserved cysteine residues in GrsA (in position 331, 377, 474 and 1065; Krätzschmar *et al.*, 1989) are not conserved in Ssv7p (Figure 6.14), making it unlikely that the N-terminus of Ssv7p performs an analogous function. A short peptide sequence at the extreme C-terminus of Ssv7p is homologous (48.6%) to Ira2p and to a lesser degree to Ira1p (Figure 6.14). The region of homology is adjacent, but not identical to the catalytic domain of GTPase activating proteins (GAPs) (Tanaka *et al.*, 1990), making it appear unlikely that Ssv7p represents a GAP protein. Secondary structure predictions of the region of homology between Ssv7p and Ira2p strongly suggests that the domains appear very similar (Figure 6.16).

```
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       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:  120.5             Length:    375
               Ratio:  0.353               Gaps:     13
Percent Similarity:  42.249   Percent Identity: 20.365


            Ssv7p (565-927) x Cdc25p (1190-1530)

                .         .         .         .         .
 565 WENLEIPCYMNCIIQCTLGTHELTQIFLDDS......YAKHININSKLGS 608
        .|.| :  |:. |: |: :|. :.:.:      :|| :  :.  |.
1190 ...NPIKCRVVNIMR.TFLTQYWTRNYYEPGIPLILNFAKMVVSEKIPGA 1235
                .         .         .         .         .
 609 KGILAKYFARLVHMMYKEQVDGSKKISISPIKFKLACGSVNSLFKTASQQ 658
     .::|.|. .:|::   ||.||.... |:|::  .   :. ..: ...|
1236 EDLLQKINEKLINENEKEPVDPKQQDSVSAVVQTTKRDNKSPIHMSSSSL 1285
                .         .         .         .         .
 659 DCQEFCQFL.LDGLH.EDLNQCGSNPPLKELSQEAEARREKLSLRIASSI 706
     .: . :.|: |. |.  |::.:. ...|. |.::       | |||.
1286 PSSASSAFFRLKKLKLLDIDPYTYATQLTVLEHD.......LYLRITMFE 1328
                .         .         .         .         .
 707 EWERFLTTDFSVIVDLFQGQYASRLKCKVCSHTSTTYQPFTVLSIPIPKK 756
      ::| :.|.:: :     |. :.  |  . .:| |.: . |::. : .|.
1329 CLDRAWGTKYCNM.....GGSPNITKFIANANTLTNFVSHTIVKQADVKT 1373
                .         .         .         .         .
 757 NSRNN...ITIEDCFREFTKCENLEVDEQWLCPHCEKRQPSTKQLTITRL 803
     .|: .   :|:.:  :|:.. ..:..    |:. .  | ..|.:|. |
1374 RSKLTQYFVTVAQHCKELNNFSSMTAIVSALYSSPIYRLKKTWDLVSTE. 1422
                .         .         .         .         .
 804 PRNLIVHLKRFDNLLNKNNDFVIYPFLL.DLTPFGANDFDGVFPPGVNDD 852
     .::|   ||.::||::...:|| |. || .:|..:. .| ||:   :.|
1423 SKDL...LKNLNNLMDSKRNFVKYRELLRSVTDVACVPFFGVY...LSDL 1466
                .         .         .         .         .
 853 ELPIRGQIPPFKYELYGVACHFGTLYGGHYTAYVKKGLKKGWLYFNDTKY 902
     .:.: |. |.|   |.. .  :.    ......|.. :.  ::.:. .:.
1467 TFTFVGN.PDF...LHNSTNIINFSKRTKIANIVEEIISFKRFHYKLKRL 1512
                .         .
 903 KPVKNKADAINSNAYVLFYHRVYGV 927
     ..:..  :|  .|.  :
1513 DDIQTVIEASLENVPHIE..... 1530
```

Figure 6.14 A: Alignment (Devereux *et al.*, 1984) of Ssv7p and Cdc25p. Vertical bars represent amino acid identities, colons represent conserved regions and dots represent similarities.

```
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       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:  115.6             Length:    450
               Ratio:  0.318               Gaps:     11
 Percent Similarity:  41.477   Percent Identity: 15.625

          Ssv7p (565-927) x Bud5p (100-538)


              .         .         .         .         .
565 ..................WENLEIPCYMNCIIQC.............. 580
                        |:: |. |:| ::.
100 LIQLVMGQDDLLFSMDDVLQEDFRIQLYLNKMLPHNSHKWQKPSPFDSAN 149
              .         .         .         .         .
581 ........TLGTHELTQIFL...........DDSYAKHINI.......NS 604
            | :.  | :::|           :.| :: |.|       :|
150 LLLNFRDWTTDNALLQELLLSYPTINKNKHKNHSVPRLIQIWVESYWQDS 199
              .         .         .         .         .
605 KLGSKGILAKYFARLV.....HMMYKEQVDG.SKKISISPIKFKLACGSV 648
    . . |:||. ::.:|.     : :: : |:   .| ....:|:.. . .
200 ETTLKDILNFWYSHLAEYYEYQELFADIVQLFINKKRTRQLKIHYIGLTD 249
              .         .         .         .         .
649 NSLFKTASQQDCQEFC.QFLLDGLHEDLNQCGS...NPPLKELSQEAEAR 694
    ..: .. .. |:::: |: :|  :.: : ||.   .. | ::.|:.
250 KEIEENKPPLDYENLFLQYEIDKTNANDELCGATDLSDLLFQWKQGELLE 299
              .         .         .         .         .
695 REKLSLRIASSIEWERFLTTDFSVIVDLFQGQYASRLKCKVCSHTS.... 740
     | :.|.:...   . :   : |:.:|:   ::..::| . .. .|
300 VEAFALNVSPWSLAKTLTLLESSLYLDIETIEFTRHFKHNDTTIDSVFTL 349
              .         .         .         .         .
741 ..........TTYQPFTVLSIPIPKKNSRNNITIEDCFREFTKC.ENLEV 779
              ||.|.  .:|. :.   |   :   ::: .:..: :| .:
350 SNQLSSYVLETTLQQTHTISYWLQVALSCLYLRNLNSLASIITSLQNHSI 399
              .         .         .         .         .
780 DEQWLCPHCEKRQPSTKQLTITRLPRNLIVHLKRFDNLLNKNNDFVIYPF 829
    :  .|. .... :   :  .:.:  .|. |. : :.:::.. . | :.
400 ERLSLPIDVKSDHLFQRLKVVVHPNNNYNVYRRTIKHIFHSQLPCVPFTS 449
              .         .         .         .         .
830 LLDLTPFGANDFDGVFPPGVNDDELPIRGQIPPF.KYELYGVACHFGTLY 878
    ||  .    .| ::.|.:.:.|: ::.  .||..:  :. |  . :::.:.
450 LLIRDITFIRDGNDTFTKDGNNVNMQKFNQITKIVAFAQYLQQKQYEDIH 499
              .         .         .         .         .
879 GGHYTA.YVKKGLKKGWLYFNDTKYKPVKNKADAINSNAYVLFYHRVYGV 927
    .:: ||  :  :: |.  .:||.| :: . . . : . .
500 CSNTTARSLLGAMIKVHTLYNDNKDRAYQVSIAKVPRLT.......... 538
```

Figure 6.14 B: Alignment between Ssv7p and Bud5p. Vertical bars represent amino acid identities, colons represent conserved regions and dots represent similarities.

```
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       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:  145.7             Length:    463
               Ratio:  0.427               Gaps:     14
 Percent Similarity: 47.319   Percent Identity: 26.814

          Cdc25p (1190-1518) x Bud5p (150-538)


            .         .         .         .         .
1190 .......................NPIKCRVVNIMRTFLTQYWTRNYY. 1213
                            |. | :  .: |  |.|.|. .|:
 150 LLLNFRDWTTDNALLQELLLSYPTINKNKHKNHSVPR..LIQIWVESYWQ 197
            .         .         .         .         .
1214 ..EPGIPLILNFAKMVVSEKIPGAEDLLQKINEKLINENEKEPVDPKQQD 1261
       |..:. ||||    :.| . : ::|:..| : :||.. . .:. .
 198 DSETTLKDILNFWYSHLAEYY.EYQELFADIVQLFINKKRTRQLKIH... 243
            .         .         .         .         .
1262 SVSAVVQTTKRDNKSPIHMSSSSL..............PSSASSAFFRL 1296
        ::.:..  :||.|:. ..  |               :.. |. :|.:
 244 .YIGLTDKEIEENKPPLDYENLFLQYEIDKTNANDELCGATDLSDLLFQW 292
            .         .         .         .         .
1297 KKLKLLDID.......PYTYATQLTVLEHDLYLRITMFECLDRAWGTKYC 1339
     |. .||:::       |:..|. ||:|| .||| |. :| :.| :
 293 KQGELLEVEAFALNVSPWSLAKTLTLLESSLYLDIETIE.FTRHF..... 336
            .         .         .         .         .
1340 NMGGSPNITKFIANANTLTNFVSHTIVKQADVKTRSKLTQYFVTVAQHCK 1389
      . ....|...:. .| |..:| .|.:.|... .      |:: ||  |
 337 .KHNDTTIDSVFTLSNQLSSYVLETTLQQTHTIS.....YWLQVALSCL 379
            .         .         .         .         .
1390 ELNNFSSMTAIVSALYSSPIYRLKKTWDLVSTESKDLLKNLNNLMDSKRN 1439
      |.|:.|:..|:..| . .| ||. . |:   .|..|:..|. ::....|
 380 YLRNLNSLASIITSLQNHSIERLSLPIDV...KSDHLFQRLKVVVHPNNN 426
            .         .         .         .         .
1440 FVKYRELLRSV..TDVACVPFFGVYLSDLTFTFVGNPDFLHNSTNIINFS 1487
     :  ||  :: :  .:::|||| ::.:.|:||.  ||..| .::.|: |:
 427 YNVYRRTIKHIFHSQLPCVPFTSLLIRDITFIRDGNDTFTKDGNNV.NMQ 475
            .         .         .         .         .
1488 KRTKIANIVEEIISFKRFHYK...........LKRLDDIQTV....... 1518
     | ..|..||.    :.. :|.           |  : .::|:
 476 KFNQITKIVAFAQYLQQKQYEDIHCSNTTARSLLGAMIKVHTLYNDNKDR 525
            .
1519 .IEASLENVPHIE 1530
      .:.|:..||::.
 526 AYQVSIAKVPRLT 538
```

Figure 6.14 C: Alignment of Cdc25p x Bud5p. Vertical bars represent amino acid identities, colons represent conserved regions and dots represent similarities.

```
          Gap Weight:  3.000      Average Match:  0.540
       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:  134.3             Length:    408
               Ratio:  0.357              Gaps:     13
 Percent Similarity:  41.341   Percent Identity: 20.670

              Ssv7p (1-390) x Grs A (45-420)
```

```
          .         .         .         .         .
  1 MEQNIISTIRDECIRHRSKYLTIAQLTAIAEAKINEFIITGKAKDQDLSS 50
    :|:.: .  .: .|   .. ||. :|.. |:.  . || .| :||  ::
 45 FEEQVSKRPNNVAIVCENEQLTYHELNVKANQLARIFIEKGIGKDTLVGI 94
          .         .         .         .         .
 51 LLDKCID....ILSIYKKNSKDIKNIISC.KNKGAMISSNS...VMIIQL 92
    :::|:||    ||.:.| .:  :.  |.: |:: . | .:|   :::.|
 95 MMEKSIDLFIGILAVLKAGGAYVPIDIEYPKERIQYILDDSQARMLLTQK 144
          .         .         .         .         .
 93 NYVYYKVIHIIVTTNIPHLSEFAKIKLHKSTSDEGNGNNNNNEFQLMNIY 142
    :.|.  :|| |  .. ..: | ..||::.:|. .. :...: .: : .
145 HLVH..LIHNIQFNGQVEIFEEDTIKIREGTNLHVPSKSTDLAYVIYTSG 192
          .         .         .         .         .
143 NT..LLETLLKDENIAKIKSFIKSSIKQTKLNHEQEECNLMRTGSYITSN 190
    .|    :|:|....|..:| |:..|:. |    |.: .. : . |: .|
193 TTGNPKGTMLEHKGISNLKVFFENSLNVT....EKDRIGQFASISFDAS. 237
          .         .         .         .         .
191 QLNSLISSSANSASSQMEILLIDIRSRLEFNKSHIDTKNI..ICLEPISF 238
     : .:: .  .:|| : |:| |. . :   . .|: |:|  |.|.|. .
238 .VWEMFMALLTGAS..LYIILKDTINDFVKFEQYINQKEITVITLPPTYV 284
          .         .         .         .         .
239 KMSYSDHDLEKKSLIT..SPNSEIKMFQSRNLFKFIILYTDANEYNVKQQ 286
       .:: |. ..||| |:.|.  : ..:: ..:| |...:.  .
285 VHLDPERILSIQTLITAGSATSPSLVNKWKEKVTYINAYGPTETTICATT 334
          .         .         .         .         .
287 SVLLDILVNHSFEKPISDDFTKIFILESGFPGWLKSNYGRKVSSSFPSNN 336
    :|  .  :.||.. . . : |.|:|::..:.  |||
335 CVATKETIGHSVPIGAPIQNTQIYIVDENLQ..LKS.............. 368
          .         .         .         .         .
337 NIKDDSVYFNGNTSGLSLQHLPKMSPSIRHSMDDSMKETASWPTPLIHLQ 386
      ::.. :  ...||. ..:.: . . .. :|:.: ....    |.. .
369 ..VGEAGELCIGGEGLARGYWKRPELTSQKFVDNPFVPGEK....LYKTG 412

387 QQQQ.... 390
    :|..
413 DQARWLSD 420
```

Figure 6.14 D: Alignment of Ssv7p x Grs A. Vertical bars represent amino acid identities, colons represent conserved regions and dots represent similarities.

```
          Gap Weight:  3.000      Average Match:  0.540
       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:   30.7             Length:     95
               Ratio:  0.370              Gaps:      2
 Percent Similarity: 48.649   Percent Identity: 20.270

            Ssv7p (845-927) x Ira2p (2160-2245)


               .         .         .         .         .
 845 ......FPPGVNDDELPIRGQIPPFKYELYGVACHFGTLYGGHYTAYVKK 888
           ..|:.:::|.: .:|..  .::        |||::. . :..
2160 SDEGLMKSVGITGQGLKVLQDIRVSLHDI.....TLYDEKRNRFTPV 2201
               .         .         .
 889 GLKKGWLYFNDTKYKPVKNKADAINSNAYVLFYHRVYGV..... 927
     :|| | :||.  . .| . |  .: :. : : :: ||::
2202 SLKIGDIYFQVLHETPRQYKIRDM.GTLFDVKFNDVYEISRIFEV 2245
```

Figure 6.14 E: Alignment of Ssv7p x Ira2p. Vertical bars represent amino acid identities, colons represent conserved regions and dots represent similarities.

```
          Gap Weight:  3.000      Average Match:  0.540
       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:   34.4             Length:   2938
               Ratio:  0.414              Gaps:      4
 Percent Similarity: 48.193   Percent Identity: 18.072

            Ssv7p (845-927) x Ira1p (1701-1850)


               .         .         .         .         .
 845 ....................................FPPGVNDDELPI 856
                                        :.. .|: |  :
1701 SFVFLRFIGPALVSPDSENIIIVTHAHDRKPFITLAKVIQSLANGRENIF 1750
               .         .         .         .         .
 857 RGQIPPFKYEL........YGVACHFGTLYGGHYTAYVKKG..... 889
     : :| . | |:        :.. :.:..: ..::|. |:.:
1751 KKDILVSKEEFLKTCSDKIFNFLSELCKIPTNNFTVNVREDPTPISFDYS 1800
               .         .         .         .         .
 890 LKKGWLYFND.TKYKPVKNKADAINSNAY....VLFYHRVYGV..... 927
     : . ::|:|: |  |.: |...  .. .:    |:: .::.||
1801 FLHKFFYLNEFTIRKEIINESKLPGEFSFLKNTVMLNDKILGVLGQPSME 1850
```

Figure 6.14 F: Alignment of Ssv7p x Ira1p. Vertical bars represent amino acid identities, colons represent conserved regions and dots represent similarities.

Figure 6.15: Chou-Fasman predictions of the regions of conserved homology between Ssv7p (A) and Ira2p (B). Both proteins have a helix - pleated sheet - helix motif, of which the sheet consits of a marginally hydrophobic region followed by two charged regions.

PLOTSTRUCTURE of: ssv7p.gcg ck: 5267

P1: Ssv7p - Saccharomyces cerevis.ae -

Chou-Fasman Prediction
June 21, 1992 22:00

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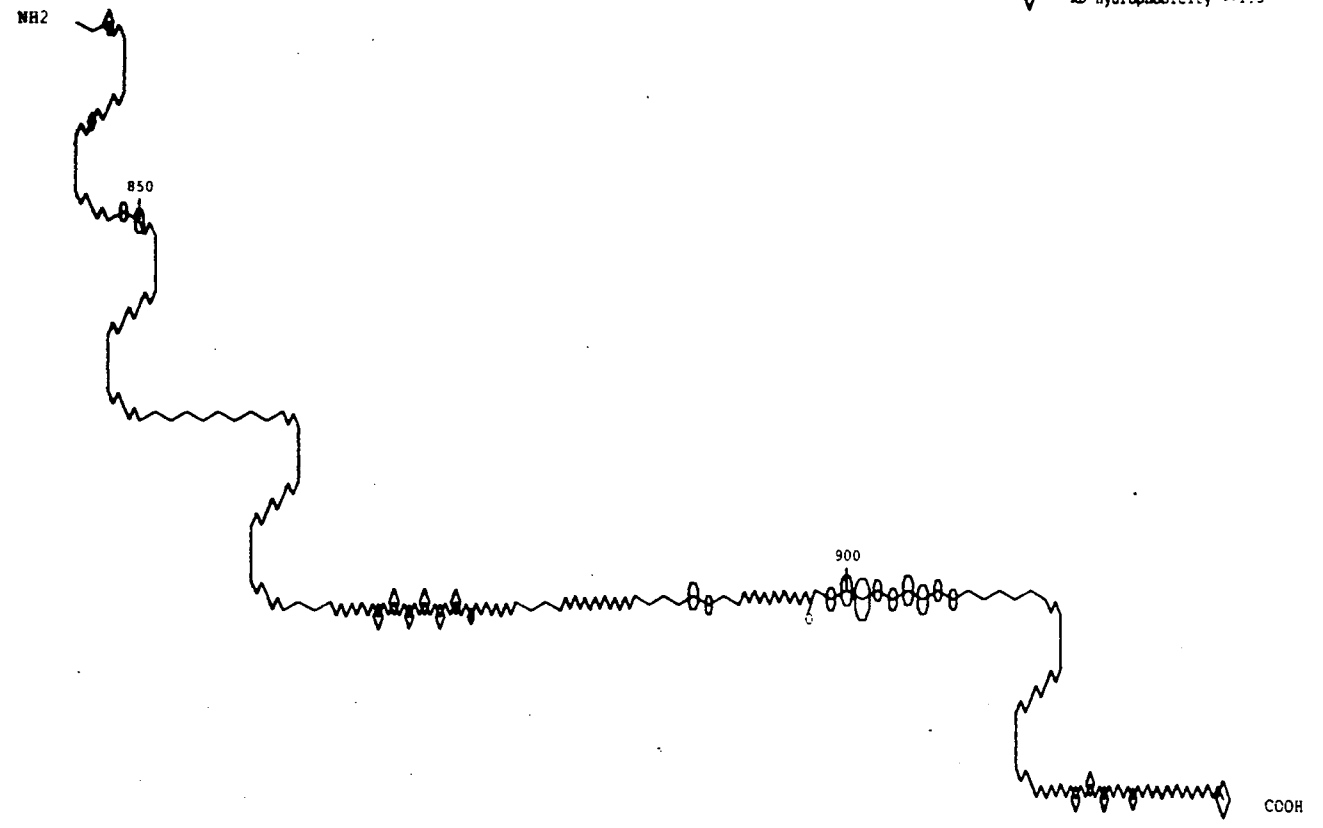
KD Hydrophobicity >=1.3

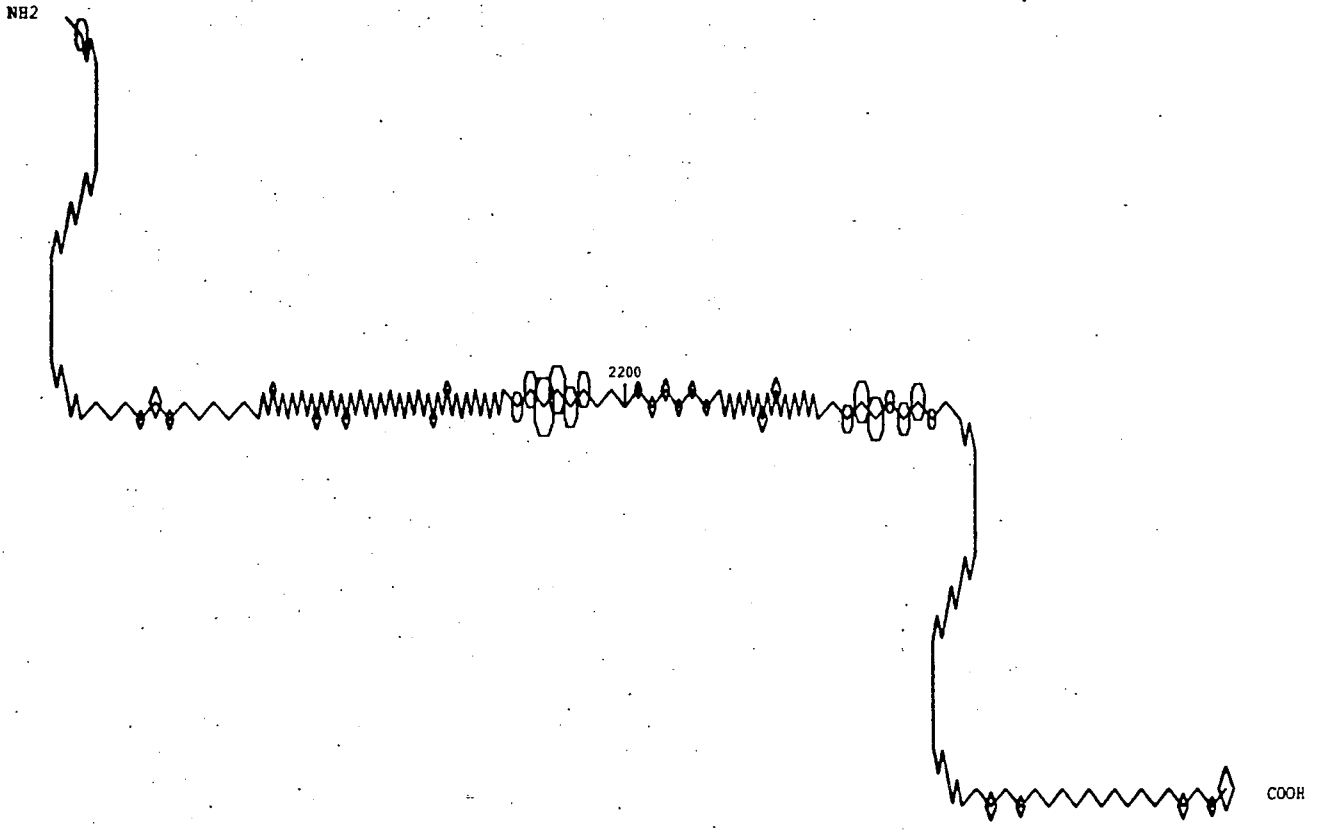


PLOTSTRUCTURE of: ira2_Yeast.sw ck: 919

P1;IRA2_YEAST - INHIBITORY REGULATOR PROTEIN IRA2 (GLC4 PROTEIN). -

Chou-Fasman Prediction
June 21, 1992 22:04

KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3



```
Ssv7p   (668)   GLHEDLNQCGSNPPLKELSQEAEARREKLSLRIASSI
Cdc25p  (1298)  LKLLDIDPYTYATQLTVLEHD        LYLRITMFE
Scd25p  (944)   ESILAVDPVLFATQLTILEHE        IYCEITTFD
Bud5p   (302)   AFALNVSPWSLAKTLTLLESS        LYLDIETIE
Ste6p   (657)   ELVLLLPPREIAKQLCILEFQ        SFSHISRIQ
Lte1p   (62)    PFILMYDSLSVAQQMTLIEKE        ILGEIDWQD
                  .* .** ..* ***.** *        *** *.. .

Ssv7p   (832)   DLTPFGANDFDGVFPPGVNDDELPIRGQ
Cdc25p  (1448)  SVTDVACVPFFGVYLSDLTFTFVGNPDF
Scd25p  (1096)  SLHSAPCVPFFGVYLSDLTFTDSGNPDY
Bud5p   (438)   FHSQLPCVPFTSLLIRDITFIRDGNDTF
Ste6p   (805)   ENCVLPCVPFLGVYFTDLTFLKTGNKDN
Lte1p   (222)   VNPLVGCVPFIVVYLSDLSANAEKKDWI
                 .. .***** *********   ** *
```

Figure 6.16: Nucleotide exchange factor consensus of Ssv7p, Cdc25p (Camonis *et al.*, 1986), Scd25p (Boy-Marcotte *et al.*, 1989), Bud5p (Chant *et al.*, 1991), Ste6p (Hughes *et al.*, 1990) and Lte1p (Wickner *et al.*, 1987). Identical and similar amino acids are indicated by an asterix or dot, respectively, using the following stringent similarity criteria: (K,R), (D,E), (N,Q), (S,T), (L,I,V), if more than three amino acids of the aligned sequences form a consensus.

6.3. Discussion

A mutation in *ssv7*-1 causes a pleitropic phenotype affecting a number of cellular processes such as nuclear and vacuolar morphogenesis, cell cycle and osmoregulation. The fact that the mutation causes an anuclear phenotype while the strain remains viable is a unique feature. Most mutants deficient in nuclear segregation (Yaffe, 1991) or in profilin (Haarer *et al.*, 1990) are conditionally lethal in that they exhibit a nuclear morphology defect at the restrictive temperature at which normal cell growth ceased. Also, these strains had a different DAPI staining pattern to the one observed in *ssv7*-1. Judged by electron microscopy, the mutant fails to assemble a nuclear envelope which is supported by the fact that the otherwise nuclear confined DAPI staining pattern is diffuse in the mutant. Compared to the nuclear morphology defect observed, the vacuolar defects like the secretion of CPY and the disrupted vacuoles are minor and probably of secondary nature. The anuclear phenotype raises the question of how the cell can compensate for the loss of the nuclear membrane. A number of cellular processes rely on temporal differential nuclear import (Nasmyth *et al.*, 1990). Also, if no nuclear membrane exists, how does correct and efficient splicing of mRNA occur? Splicing occurs in the nucleus and it has been suggested that the nuclear envelope prevents unspliced RNA from escaping into the cytoplasm (Michaud and Goldfarb, 1991). On the other hand, nuclei of nearly all eukaryotic cells vesicularize during mitosis (Fry *et al.*, 1976; Lucocq *et al.*, 1987; Zeligs and Wollman, 1979) and the presence of chromosomes in the cytoplasm alone seems not to affect viability. Consequently, the phenotype of the *ssv7*-1 mutant argues that yeast cells are viable without a nucleus under laboratory conditions. This is even more bizarre when one considers that the yeast nucleus does not vesicularize before division (Yaffe, 1991). Alternatively, the nucleus in *ssv7*-1 could be fragmented into micronuclei, where each chromosome is surrounded by a miniature nuclear envelope. This would raise the problem of how partitioning of the chromosomes takes place during mitosis and requires spindle pole bodies for each micronucleus. Lastly one possibility accounting

for the anuclear phenotype remains. *ssv7*-1 strains could have morphologically intact nuclei, but the mutation affects the nuclear stability during both formaldehyde and glutaraldehyde fixation procedure. This later possibility seems unlikely as DAPI staining of live cells shows the same effect (C.A. Hartley and M.D. Watson, unpublished results).

Judged from the growth rate of the *ssv7*-1 mutant it appears that the mutant is viable and not too significantly affected by the mutation. However several regulatory processes are severely affected, including the cell cycle. While in wild type the cell growth and budding are coupled, the *ssv7*-1 strain uncouples cell growth from budding, typical for a limitation in the division cycle (Cross *et al.*, 1989). Although the exact defect in the cell cycle was not further analysed, an explanation consistent with all available evidence is that cell size is unlinked to START thus allowing uncontrolled growth. From the data obtained it it is impossible to judge which is the primary defect in the mutation, although the aberrant nuclear phenotype is a good candidate, because all other phenotypes can be explained as a consequence of a regulatory defect resulting from the dilution of transcriptional regulators by the cytoplasm. Other regulatory pathways were not studied in detail but it appears that at least adenine biosynthesis is also affected by the mutation because the *ade2 ssv7*-1 strain MLY1511 accumulates an excess of the red adenine fluorochrome giving it a bright red appearence.

It was decided to study the mutation at the molecular level by cloning and sequencing its corresponding gene. While the cloning was successful, some standard genetic tests could not be performed because the necessary plasmid constructs for single step gene disruption and integrative mapping were not available at this time. Therefore no further data are available whether the gene *SSV7* is the wild type copy of *ssv7*-1 or a single copy suppressor (see Chapter 5), and whether the cloned gene is essential for cell growth. Sequence analysis established that the gene has a single open reading frame encoding a 927 amino acid protein with a predicted molecular mass of 105,000 Da. It has no extensive secondary structure in the form of a long α helix, making it unlikely that it represents an intermediate filament type protein. Sequence comparison revealed that the N-terminal domain of Ssv7p has homology to gramicidin S I synthetase, a

protein involved in a racemisation reaction (Krätzschmar *et al.*, 1989). The significance of this homology is not understood, but may be indicative for either similar substrate binding (phenylalanine) or the catalysis of a similar reaction. The region of limited homology is separated from another functional region by approximately 200 amino acids with no known homology to other proteins. The C-terminal domain of the protein has significant homology to the consensus sequence of GDP-GTP exchange factors (Boy-Marcotte *et al.*, 1989; Broek *et al.*, 1987; Camonis *et al.*, 1986; Chant *et al.*, 1991; Hughes *et al.*, 1990; Powers *et al.*, 1991; Wickner *et al.*, 1987). It has previously been suggested that the structural similarities between the putative nucleotide exchange factors could reflect general features of the mechanism of nucleotide exchange (Powers *et al.*, 1991). As yet, their mechanism of nucleotide exchange is not known. Cdc25p, Scd25p and Ste6p are true nucleotide exchange factors for *RAS* proteins (Broek *et al.*, 1987; Camonis *et al.*, 1986; Chant *et al.*, 1991; Hughes *et al.*, 1990; Powers *et al.*, 1991) and actually mediate nucleotide exchange. Other similar factors, such as Bud5p or Lte1p, bind to *RAS* proteins but are thought not to directly mediate exchange (Powers *et al.*, 1991). Since Ssv7p is closer related to Cdc25p than to Bud5p it appears plausible that Ssv7p is a true nucleotide exchange factor. The limited homology and structural similarity to a short region of the GAPs Ira1p and Ira2p, not identical to their catalytic domain (Tanaka *et al.*, 1990), suggests that they share a common function to both GAPs and nucleotide exchange factors.

What is the intracellular target of Ssv7p? The answer is unknown, but speculation suggests that it might function in the organization of the nucleus or cytoskeletal features. Nucleotide exchange factors activate corresponding GTPases by exchanging a GDP with an GTP (Engelberg *et al.*, 1990). The GTPase activated by Ssv7p has to be identified as has its exchange activity. Judging from the appearance of the *ssv7*-1 mutation, it might control a nuclear envelope function. Postmitotic nuclear assembly in mammals occurs by GTP dependent vesicle fusion on chromatin condensation points (Boman *et al.*, 1992; Newport and Dunphy, 1992). The GTPase involved in this assembly might be regulated by an exchange factor similar to Ssv7p. On the other hand, the yeast nucleus does not vesicularize during mitosis, and thus Ssv7p might function in

its maintenance. Alternatively, Ssv7p could stimulate the polymerization status of cytoskeletal elements, which when disrupted leads to fragmentation of nuclei as well as vacuoles.

The findings described in this chapter provide starting material for future investigations into the nature of the nuclear defect observed in the *ssv7*-1 mutant as well for the analysis of the Ssv7 protein at the molecular level. Site directed mutagenesis might help to define regions involved in GTPase binding, nucleotide exchange and putative upstream or downstream effector regions. At the same time it can be addressed what effect a complete or partial deletion of the coding sequence has on nuclear morphology. PCR mediated cloning of the *ssv7*-1 mutant allele will help to indentify the location of the mutation and thus further domains. The isolation of suppressors and their analysis as well as biochemical cross-linking experiments will enable the identification of upstream and downstrem elements. Immunolocalization of the Ssv7 protein will locate its action to a compartment. Assuming that nuclear assembly will occur in the presence of wild type Ssv7p, an *in vitro* system can be developed that allows the dissection of nuclear assembly as well as the analysis of Ssv7p action. Epistasis analysis of a *ssv7* mutant could determine the location of the cell cycle defect. Of interest is also the nature of osmosensitive growth in the *ssv7*-1 mutant. Lastly the mutant was isolated on the basis of osmosensitivity and aberrant vacuolar morphology, although at present it appears that the osmoregulatory defect is a consequence of the nuclear defect.

Although part of the genetic analysis of *SSV7* could not be performed because the necessary recombinant plasmids were not available, its outcome would not have furthered the knowledge about *SSV7* and its direct and indirect role in nuclear morphogenesis. Whether Ssv7p is defective in the formation of a nuclear envelope or can suppress a defect of another mutation, cannot be decided at present. What is unambiguous is the fact that a putative nucleotide exchange factor participates in the assembly/maintenance of nuclear integrity.

CHAPTER 7

CHARACTERIZATION OF *ARO7-OSM2*

7.1. Introduction

OSM2, a gene required for osmohomeostasis in yeast, has been mapped near to the *ARO7* locus (Singh and Sherman, 1978). Subsequent genetic analysis showed that *OSM2* and *ARO7* are identical (Ball *et al.*, 1986). *SSV15*, a gene involved in osmohomeostasis and vacuole biogenesis in yeast, is allelic to the *ARO7* locus by virtue of its complementation pattern. A mutation in the *SSV15* locus leads to the partial secretion of the vacuolar enzymes such as carboxypeptidase Y (CPY) and α-mannosidase, and the absence of any visible vacuole (Latterich and Watson, 1991; see Chapter 4).

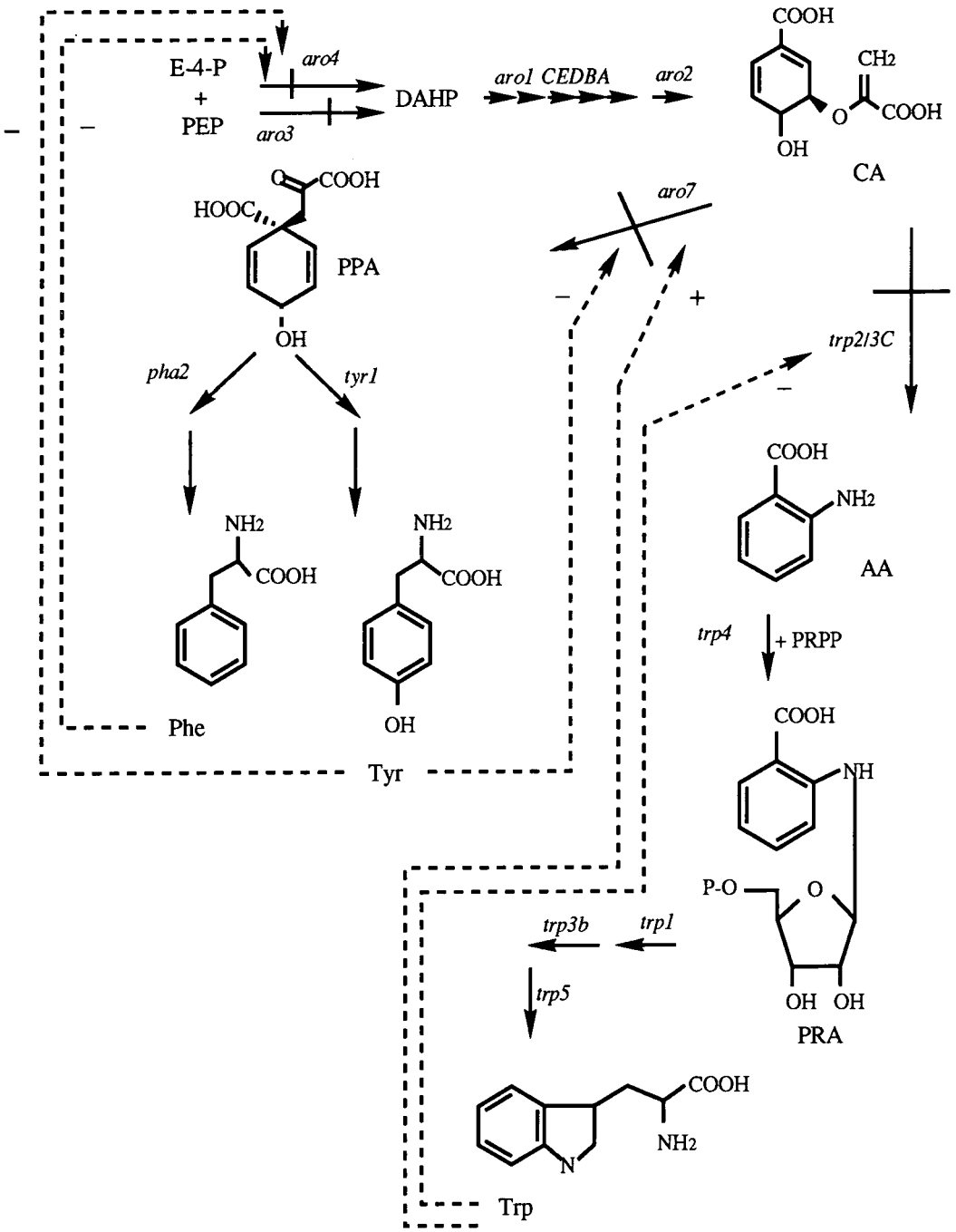
ARO7 is located on chromosome XVI (Mortimer *et al.*, 1989) and encodes chorismate mutase (E.C. 5.4.99.5) (Kradolfer *et al.*, 1977; Schmidheini *et al.*, 1989). The enzyme is required to catalyze the reaction from chorismate to prephenate in the aromatic aminoacid biosynthesis pathway in the yeast *Saccharomyces cerevisiae* (Braus, 1991; see Figure 7.1). Its structural gene *ARO7* has been cloned, and its nucleotide sequence determined (Ball *et al.*, 1986; Schmidtheini *et al.*, 1989). It encodes a protein of 256 amino acids, which has no recognizable signal peptide and which shows no significant homology to the corresponding domains of the two bifunctional *E. coli* enzymes required for the same biosynthetic step (Braus, 1991).

Aro7p has been extensively characterized in terms of its biosynthetic properties and its regulation (Braus, 1991). The enzyme, a dimer consiting of two identical 30 kDa subunits, is feedback inhibited by tyrosine representing one of its end products of the biosynthetic branch and also activated by tryptophan, the other end product of the branch (Kradolfer *et al.*, 1977). Gene expression of the *ARO7* gene (Schmidheini *et al.*, 1989; Schmidheini *et al.*, 1990b) as well as mRNA stability of the *ARO7* transcript

(Braus, 1991) are not subject to feedback regulation by metabolic precursors or end-products. The precise mechanism of stimulation or inhibition of the enzyme is unknown, except that tryptophan can allosterically activate chorismate mutase 10 fold while tyrosine can inhibit the enzyme 10 fold (Braus, 1991). Constitutively activated choismate mutase mutants are irresponsive to inhibition or activation by its two effectors (Schmidheini *et al.*, 1990a). Cloning of the chorismate mutase gene and the constitutively activated gene revealed that a point mutation resulting in an amino acid substitution from threonine to isoleucine in position 226 of the peptide sequence is responsible for the constitutive active enzyme (Schmidheini *et al.*, 1989). The constitutively active enzyme will result in a depletion of the chorismate pool. The active enzyme converts chorismate directly and irreversibly to prephenate without any detectable intermediates (Schmidheini *et al.*, 1990a). Kinetic data have shown that the dimerized enzyme is in equilibrium with the monomeric form, where the monomers have a lower affinity for the ligands (revied in Braus, 1991). The role of chorismate mutase in osmotic stability is not known.

Since the gene corresponding to the *ARO7-OSM2* locus has been cloned twice and sequenced before, the cloning and sequencing of the *SSV15* gene by complementation of the *ssv15*-1 allele was omitted, but the phenotype of the original *osm2* mutation (here after referred to as *aro7*) was closer examined to understand its function in the wild type yeast cell. An *aro7*-1 mutant like the previously isolated *ssv15*-1 mutant (MLY0716; MLY1502) was found to confer multiple mutant phenotypes, namely osmosensitivity, inability to grow on medium lacking tryptophan and phenylalanine, secretion of vacuolar proteinases and a severe defect in vacuole biogenesis. It was decided to use the *aro7*-1 allele rather than the *ssv15*-1 allele for a phenotypic analysis because the latter allele tended to revert to the wild type at a higher frequency than did the *aro7*-1 allele.

Figure 7.1: Biosynthesis of aromatic amino acids and feedback regulation of the enzymes in *S. cerevisiae*. CA: chorismate; AA: anthranilate; PPA: prephenic acid; *ARO3/ARO4*: 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (EC 4.1.2.15); *ARO1C*: 5-dehydroquinate synthase (EC 4.6.1.3); *ARO1E*: 3-dehydroquinate dehydratase (EC 4.2.1.10); *ARO1D*: dehydroshikimate dehydrogenase (EC 1.1.1.25); *ARO1B*: shikimate kinase (EC 2.7.1.71); *ARO1A*: 5-enolpyruvylshikimate 3-phosphate synthase (EC 2.5.1.19); *ARO2*: chorismate synthase (EC 4.6.1.4); *ARO7*: chorismate mutase (EC 5.4.99.5); *PHA2*: prephenate dehydratase (EC 4.2.1.51); *TYR1*: prephenate dehydrogenase (EC 1.3.1.13); *TRP2*: anthranilate synthase (EC 4.1.3.27); *TRP3C*: glutamine amidotransferase; *TRP4*: anthranilate phosphoribosyltransferase (EC2.4.2.18); *TRP1*: PRA isomerase; *TRP3B*: InGP synthase (EC 4.1.1.48); *TRP5*: tryptophan synthase (EC 4.2.1.20). The *ARO1* gene product is a pentafunctional enzyme catalyzing five individual enzymatic reactions. The *TRP3* gene product is a bifunctional enzyme encoding two enzyme activities.


E-4-P
+
PEP
aro4
aro3
DAHP
aro1 CEDBA
aro2
COOH
CH2
O
COOH
OH
CA
HOOC
O
COOH
PPA
OH
aro7
pha2
tyr1
trp2/3C
NH2
COOH
NH2
COOH
OH
Phe
Tyr
NH2
COOH
AA
trp4
+ PRPP
COOH
NH
P-O
O
OH OH
PRA
trp3b
trp1
trp5
COOH
NH2
N
Trp

7.2 Results

7.2.1. Electron Microscopic Examination of the *aro7*-1 Mutant

Electron microscopic examination of the *aro7*-1 mutant using two different fixation protocols shows that the mutant has no visible intact or disrupted vacuoles (Figure 7.2). Whereas the wild type strain SEY6210 always has one to three large, dark staining vacuoles present (Figure 7.2 A and C), the *aro7*-1 mutant strain has no detectable vacuoles present in more than 100 individual cells examined under the electron microscope (Figure 7.2 B and D). The results are consistent with different staining procedures, making the artefactual absence of vacuoles in the *aro7*-1 mutant unlikely. While other previously isolated vacuolar class C mutants did not have distinct vacuoles, they did accumulate some small membrane enclosed vesicles whose periphery stained like vacuoles but whose center was electron opaque (Chapter 4). No similar structurs could be observed in the *aro7*-1 mutant indicative of a different defect than in other class C mutants. No other abnormal subcellular structures were observable in this strain.

7.2.2. Indirect Immunofluorescence

Since a small fraction of an *aro7*-1 culture has normal vacuoles as determined by Normarski contrast microscopy, its vacuolar morphology was examined by indirect immunofluorescence microscopy with antibodies against two vacuolar membrane proteins, namely alkaline phosphatase (Pho8p) and the 60 kDa subunit of the vacuolar ATPase (60 K) (Roberts *et al.*, 1991). Pho8p is not localized in a large vacuolar compartment, as is the case for the wild type, but seems to accumulate in numerous small vesicles in the cytoplasm, as is characteristic for *ssv* or *vps* class C mutants lacking normal or disrupted vacuoles (Figure 7.3). Using an antibody against 60 K, the immunofluorescence pattern reveales the presence of the vacuolar ATPase subunit as is typical for normal cells with a mature vacuole, but only in cells which appear to have a mature vacuole under Normarski contrast microscopy also. A total of approximately

20% of all cells do have a visible vacuole by this method, the rest having a class C morphology only. It was not tested whether the cells with morphologically mature vacuoles were revertants or part of a mixed vacuolar phenotype population. The osmosensitive phenotype in this mutant reverts quite readily to the wild type, and stock cultures had to be purified and tested for osmosensitivity before each use.

7.2.3. Missorting of Carboxypeptidase Y

Immunoprecipitation of the soluble vacuolar enzyme CPY in both intracellular and extracellular fractions provides evidence that a significant amount (approximately 20%) of the 68 kDa CPY precursor is secreted in the *aro7*-1 mutant rather than correctly targeted to the vacuole as in the wild type, whereas the remaining intracellular CPY fraction is processed to the 61 kD mature form of the vacuolar enzyme (Figure 7.4). The maturation pattern suggests that *aro7*-1 mutants must have an intracellular compartment where *PEP4* dependent maturation of p2CPY to mature CPY can occur.

7.2.4. Examination of Other Mutants in the Aromatic Amino Acid Pathway

Two mutants in the aromatic amino acid biosynthesis pathway, *aro3*-2, *aro4*-1 defective in both isoenzyme activities of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (EC 4.1.2.15) and in *aro2* defective in chorismate synthase (EC 4.6.1.4) activity were tested for their vacuole biogenesis related phenotypes. Both mutants affect steps prior to chorismate mutase and thus do not synthesize chorismate. Compared to *aro7*-1 and the wild type both strains were able to maintain growth on YPD supplemented with 1.5 M NaCl. Vacuolar morphological analysis by localizing Pho8p and the 60 kDa subunit of the vacuolar ATPase did not reveal any aberrant vacuolar morphology in both mutants. Also immunoprecipitation of intracellular and extracellular CPY did not detect any extracellular CPY (results all not shown). All intracellular CPY was matured to the 61 kDa CPY. Thus mutants defective in other enzymes of the same biosynthetic pathway did not have the same phenotype as the *aro7*-1 mutant.

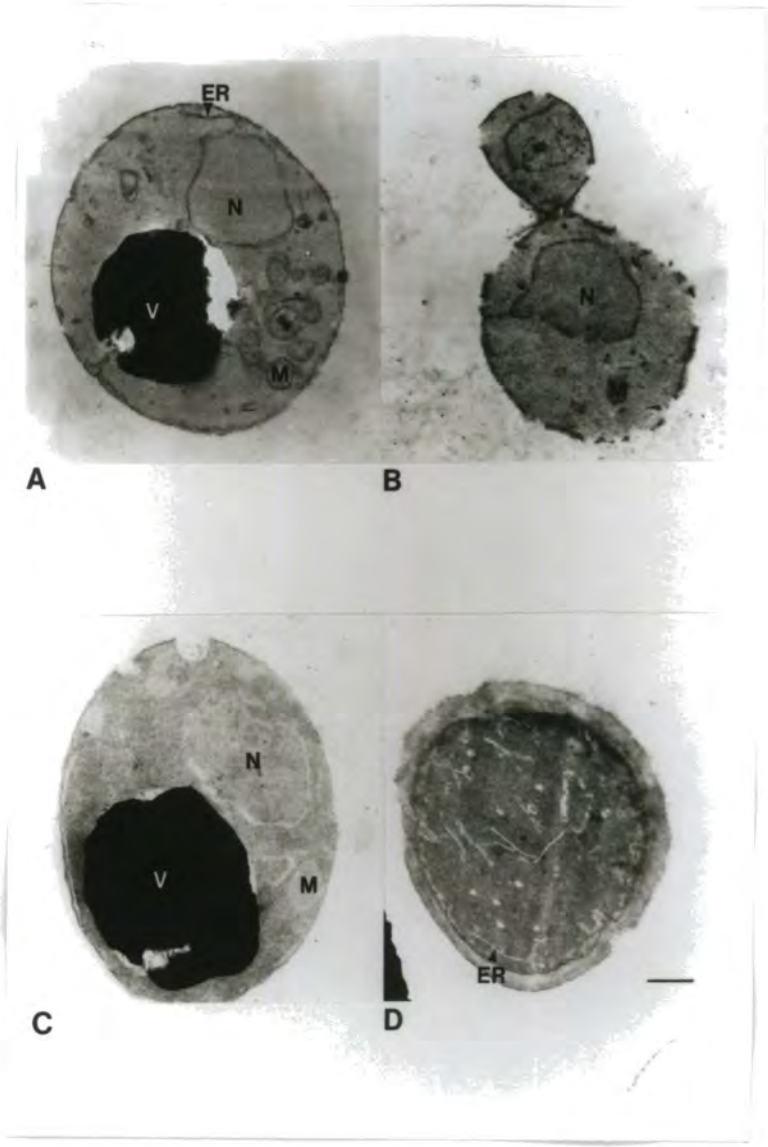


Figure 7.2: Electron micrographs of SEY6210 (wild type) and B-4164 (*aro7*-1), using two different fixation protocols. The wild type strain has a clearly visible electron dense vacuole (A) and (C), which cannot be found in the *aro7* mutant strain (B) and (D). Cells in (A) and (B) were fixed using the ferricyanide reduced osmiumtetroxide - thiocarbohydrazide - reduced osmiumtetroxide protocol to specifically stain double membranes and carbohydrate structures. Cells in (C) and (D) were fixed and stained according to the method by Byers and Goetsch. ER, endoplasmic reticulum; N, nucleus; M, mitochondrium; V, vacuole. Bar = 0.5 μm.

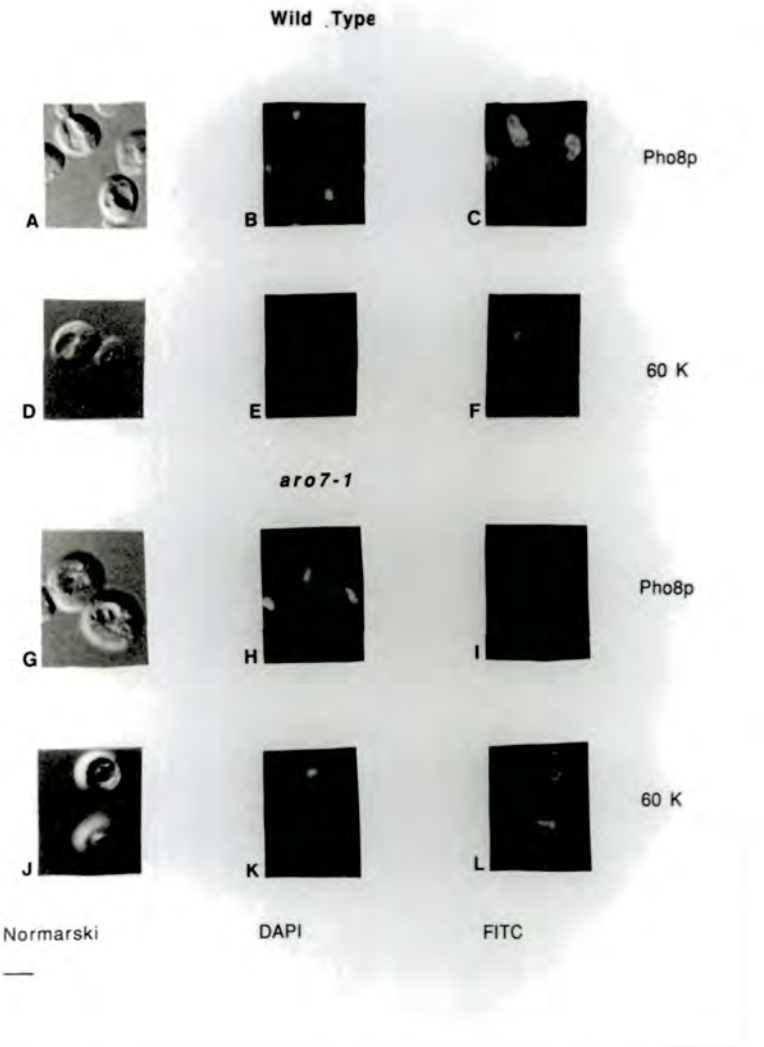


Figure 7.3: Indirect immunofluorescence of SEY6210 (wild type), and B-4164 (*aro7*-1), using primary antibodies against Pho8p and 60 K. In the wild type (A-F) vacuoles are visible under Normarski contrast (A and D), and vacuolar membrane proteins Pho8p and the 60K subunit of the vacuolar ATPase are highlighting a compartment that corresponds to the yeast vacuole (C and F, respectively) which differ from the nucleus (B and E). In the *aro7* mutant strain B-4164, vacuoles are detectable by Normarski contrast microscopy only in some cells (G and J) and are recognized by the anti-60K antiserum (L), but not by the anti-Pho8p antiserum (I). The DAPI staining of nucleus and mitochondria (H and K) served as a control. Bar = 1 μm.

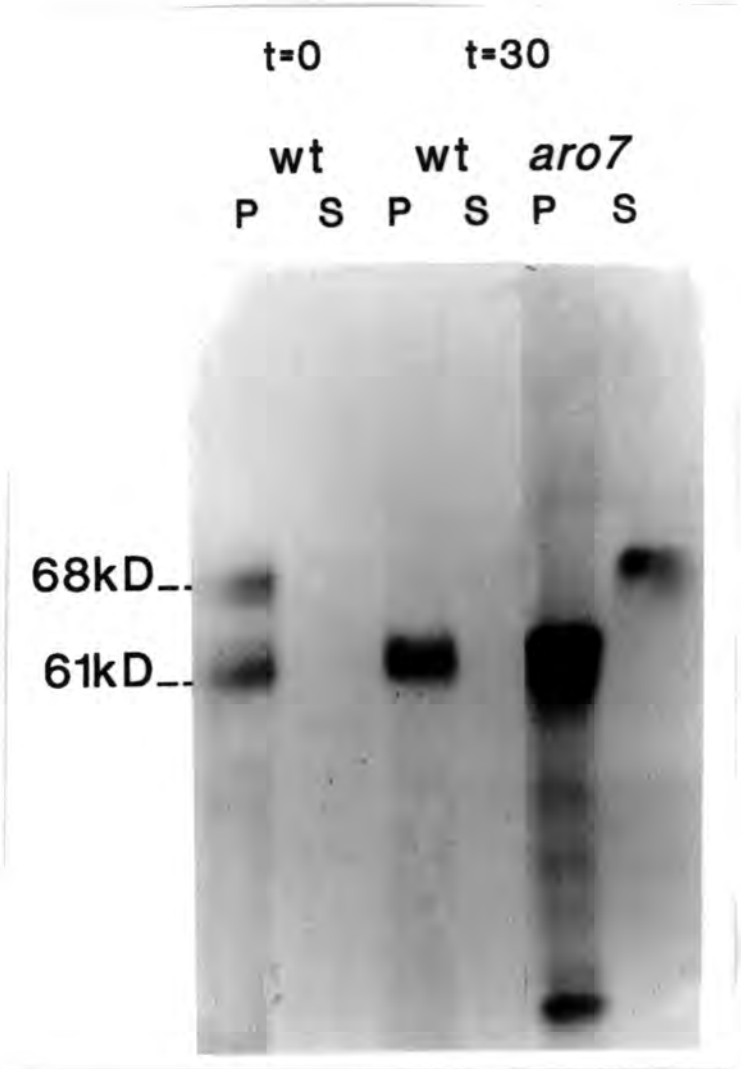


Figure 7.4: Secretion of CPY in SEY6210 (wild type) and B-4164 (*aro7-1*). Lanes P and S of each strain show the immunoprecipitation of CPY from sphaeroplasts and culture supernatant respectively. After 0 minutes chase, equal amounts of the 68 kDa carboxypeptidase Y (CPY) precursor and 61kD mature CPY are present in the sphaeroplasts. After a chase of 30 minutes with cold methionine, all of the precursor has been converted into the 61kD mature form. In the wild type none of the CPY is secreted, whereas in the *aro7* mutant strain part of the CPY precursor is secreted (lane S) and the remaining intracellular form is matured (lane P).

7.3. Discussion

The *aro7* mutant has a vacuolar morphology phenotype characteristic of class C *vps* mutants described before (Banta *et al.*, 1988), and also a significant *vps* mutant phenotype in that it secretes a considerable amount of the precursor of the vacuolar proteinase CPY. Based on indirect immunofluorescence microscopy studies of two vacuolar membrane markers, alkaline phosphatase and the 60 kDa subunit of the vacuolar ATPase, a sub-population of cells present in a *aro7*-1 culture has a vacuolar compartment as defined by localization of the 60 kDa subunit of the vacuolar membrane ATPase to a large spherical compartment representing a vacuole. Alkaline phosphatase/fructose-2,6-bisphosphate 6-phosphatase (Pho8p) appears to accumulate in vesicles in all cells examined, which is typical for a class C phenotype. *aro7*-1 differs from other *ssv* and *vps* mutants in that it is able to mature the intracellular fraction of CPY, which has not been observed with other vacuolar class C mutant strains Robinson *et al.*, 1988; Chapter 4). These results cannot be explained with the current concept of proteinase A dependent maturation of CPY, since this process requires a fully functional vacuolar compartment (Klionsky *et al.*, 1990), and all previously identified mutants lacking an electron microscopically visible vacuole are unable to mature intracellular CPY (Latterich and Watson, 1991; Robinson *et al.*, 1988). One plausible explanation is that the *aro7* mutation affects directly or indirectly the assembly of a structurally intact vacuole but not its biochemical function. The vacuole may be vesicularized in the mutant as is the Golgi apparatus in wild type yeast (Fransuzoff *et al.*, 1991a). The vacuolar biochemical functions could be maintained within the vesicles. Alternatively, the mixed vacuole phenotype might cause the missorting phenomenon of the vacuolar proteinase CPY in the mutant. In a mixed population some cells have intact vacuoles and are able to correctly sort and mature the CPY. Cells in the same population that do not have vacuoles will secrete all of the CPY due to the absence of the receptor compartment. Taken together, the culture will have the secreted precursor CPY present, as well as matured CPY in a vacuole expressing sub-population.

However the proportion of cells secreting CPY does not correspond to the relatively small proportion of cells with vacuole like compartments as seen by 60 kDa immunofluorescence.

Considering the results obtained by immunolocalization of 60 K, it appears that in the *aro7* mutant the structural organelle "vacuole" exists in a sub-population, but it seems to selectively accumulate certain vacuolar proteins, and not others. Considering also the results obtained by electron microscopy, and the maturation of the intracellular fraction of CPY, some mutant cells appear to have a partially functioning organelle that is competent for some proteolytic processing, but not for other functions such as osmoregulation, delivery of Pho8p and some factors that cause elctron dense vacuoles with the staining methods employed. The fact that the majority of *aro7*-1 cells do not have any visible vacuole by any criterion is interesting because the gene product of *ARO7* is a biosynthetic enzyme in aromatic amino acid biosynthesis.

It is startling that a mutation in a gene encoding a biosynthetic enzyme has such severe pleiotropic effects on protein targeting and especially organelle biogenesis even in presence of the end metabolites of the pathway affected. The question is whether the reaction product(s) or the enzyme *per se* are required to maintain vacuole structure and function. The lack of available tyrosine and/or phenylalanine, or an excess of tryptophan could lead to a stall in the synthesis of a protein required for organelle biogenesis. The wild type phenotypes are not restored when the mutant is grown in medium supplemented with an excess of aromatic amino acids (data not shown). This makes it appear less likely that a lack of aromatic amino acids alone is responsible for the multiple phenotypes associated with the mutant. Also, mutants in *aro3*, *aro4* and in *aro2*, which synthesize precursors to chorismate, do not have any of the defective vacuolar phenotypes observed in *aro7* strains. An alternative explanation is that Aro7p is a bifunctional protein and carries out another function related to vacuole biogenesis, althogh there as yet is no experimental evidence to support this hypothesis. It has been demonstrated recently that Aro1p associates *in vitro* with microtubules (Barnes *et al.*, 1992). This could also be the case for Aro7p. In strains lacking a functional Aro7p the

mutation could possibly affect microtubular function as a result. *In vitro* site directed mutagenesis of *ARO7* might help in the elucidation of its function.

Amino acid pools in some organisms increase if the osmotic strength of the medium rises (Csonka, 1989). In yeast phenylalanine and/or tyrosine might be sequestered in a similar process. Alternatively, chorismate, which could accumulate in *aro7* mutants, might have a deleterious effect on osmoregulation and vacuole biogenesis directly, or by aiding the excessive accumulation of biochemical intermediates in other branches of the pathway. The involvement of aromatic amino acids in osmoregulation has not been demonstrated thus far. It appears unlikely that a lack of tyrosine and phenylalanine or an excess of tryptophan is responsible for the pleiotropic vacuolar defects associated with *aro7*, because an excess of all aromatic amino acids individually or alone does not restore vacuoles in *aro7* nor does it lead to vacuolar morphology changes in the wild type. Chorismate is an intermediate in ubiquinone, p-aminobenzoate and vitamin K biosynthesis. Ubiquinone is a electron acceptor in mitochondria, p-aminobenzoate a precursor in folic acid biosynthesis, and vitamin K a cofactor in γ-carboxylation reactions. An excess of any of these components due to accumulation of chorismate could shift the intermediate equilibrium in a way that factors influencing vacuole morphology are expressed or repressed. The effect of an excess of chorismate in the wild type was not examined for financial reasons.

This example of an apparent involvement of a biosynthetic enzyme in sorting of vacuolar proteins and organelle biogenesis stresses the complexity and plasticity of the processes required for protein sorting. If the fidelity of cellular processes, such as the biosynthesis of primary or secondary metabolites is disturbed, it can have a series of secondary effects on apparently unrelated processes. This consideration is especially important in interpreting mutational analysis of cellular events, since the mutant phenotype selected for can be the consequence of a mutation in an functionally unrelated gene which changes the fidelity of key cellular processes, or the inactivation of a multifunctional enzyme.

CHAPTER 8

FINAL DISCUSSION

8.1. Osmohomeostasis and the Vacuole

This study addressed the proposed function of the yeast vacuole in cellular osmohomeostasis. The evidence obtained so far suggests that some vacuolar function is required for intracellular osmohomeostasis. The nature of the osmoregulatory mechanism could not be elucidated, although by isolation and characterization of *ssv* genes and lastly the development of *in vitro* reconstitution of the osmoregulatory vacuolar function this can be accomplished. The *ssv* mutants isolated in this study were defective in an immediate osmoregulatory response, which is different to the adaptive osmoresistance dependent on the accumulation of glycerol (Blomberg and Adler, 1989). Assuming that the osmosensitive phenotype is primarily caused by a vacuolar defect, the vacuole must allow survival to otherwise lethal osmotic stress by regulating the cytoplasmic water potential before other slower adaptive masures can be taken. One likely possibility is that osmotic gated vacuolar membrane channels open during osmotic shock to balance the osmotic loss of water from the cytoplasm. No such channels have been identified in *S. cerevisiae*. The presence of a hydrostatic and osmotic pressure activated channel in the plant vacuolar membrane has been demonstrated (Alexandre and Lassalles, 1991). This channel is activated by both hydrostatic pressure on either side, or an osmotic gradient, using sorbitol as an inducing agent. The channel was gated under *in vivo* conditions by a sole change in osmolality, without the need of a turgor change. The channel appears to regulate water flux between cytosol and the vacuole, and is also permissive to K^+ and to a lesser extent to Cl^- ions. A similar mechanism of osmotic potential gating could exist in yeast vacuoles, which is consistent with all preliminary findings in this and other studies. A non-functional water potential regulating channel caused by mislocalization of some component due to a

vacuolar targeting defect or due to a mutation in a gene(s) encoding the channel protein(s) could explain the mutants failure to immediately adapt to osmotic stress even before the osmoprotectant glycerol and other *de novo* synthesized proteins can adapt the yeast to the new environment. The fact that some newly synthesized gene products together with glycerol are required for long term survival under osmotic stress (Blomberg and Adler, 1989) does not contradict these considertions. Patch-clamp studies on the isolated vacuolar membrane from yeast analogous to the approach described above (Alexandre and Lassalles, 1991) could demonstrate the presence of a similar osmoregulated ion-conducting channel. The proposed existence of an immediate osmoregulatory mechanism will prevent immediate cell death under unfavourable conditions and allow secondary osmotic responses to be initiated. In the unicellular alga *Dunaliella salina* the primary osmotic response consists of swelling or shrinking of the cell which is not surrounded by a rigid cell wall due to very rapid water fluxes. A secondary metabolic response is initiated which is mediated by a chloroplastic P_i / triose-phosphate shuttle, activating or deactivating chloroplastic enzymes in either starch or glycerol synthesis according to the osmolarity of the surrounding medium (Bental *et al.*, 1990). Yeast vacuoles respond with swelling or contraction dependent on the water pressure in the surrounding environment hinting that at least the vacuole can balance turgor pressure. It appears that in different organisms at least two osmotic responses exist, an immediate response to mediate turgor adaptation and a secondary metabolic response to adapt to the change in osmolarity and provide an osmotic balance to allow normal metabolic processes to occur.

The molecular analysis of *SSV7*, the gene probably corresponding to the *ssv7*-1 locus, revealed that it represents a nucleotide exchange factor by sequence homology. Ssv7p could transmit a signal induced by osmotic changes to a thus far unidentified GTPase and initiate a GTP-binding protein mediated immediate osmoregulatory response. Considering the anuclear phenotype observed in the *ssv7*-1 mutant, this hypothesis appears unlikely. Rather, the osmosensitivity and vacuolar morphology defect might result from deregulated transcription as an immediate result of the absence of a nuclear membrane. The osmosensitive phenotype might actually represent a

conditional phenotype, similar to temperature sensitivity, and not an osmoregulatory defect. This finding somewhat questions the isolation procedure of the *ssv* mutants, because *ssv7*-1 was a "typical" *ssv* mutant in that it was osmosensitive, had a vacuolar morphology defect and also mislocalized a fraction of its p2CPY to the cell surface. Other genes in similar pathways could affect vacuole morphology and osmoregulation.

8.2. Protein Traffic to the Vacuole

The analysis of *vps* mutants will aid the elucidation of the sorting mechanisms responsible for the recognition of p2CPY and the immediate processes involved in retrieval of the protein from the Golgi compartment where sorting occurs. The approach taken by two research groups to isolate mutants involved in vacuolar sorting are based on the mislocalization of p2CPY (Rothman *et al.*, 1986) or a ppCPY-invertase hybrid protein (Bankaitis *et al.*, 1986). A total of approximately 49 different complementation groups were obtained affected in the localization of a number of vacuolar proteins (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). This has led to the conclusion that their putative gene products are involved in the sorting of all soluble vacuolar enzymes. The finding that Vps35p is specifically involved in the sorting of CPY but not other vacuolar proteins as are a number of *ssv* genes is a first indication that both approaches will have isolated mutants mainly in the specific recognition of CPY but not other soluble vacuolar proteins, and components commonly involved in the early sorting processes. This is corraborated by the fact that most *vps* mutant alleles secrete p2CPY but not always PrA or PrB to the same degree. Secondly, proteins leaving the Golgi in vacuole destined transport vesicles will not be able to fuse back with the Golgi apparatus, assuming unidirectionality of specific transport events, resulting in accumulation of these vesicles in the cytoplasm (Baker *et al.*, 1990; Bourne, 1988; Rothman and Orci, 1992). The unidirectionality argument is favoured by considering the findings derived from *sec* mutants. For instance *sec1* mutants will accumulate secretory vesicles but not ER or Golgi under restrictive conditions indicative of unidirectionality or kinetically favoured transport into the forward direction (Kaiser and Schekman, 1990). Thus all

mutants defective in the targeting and fusion of Golgi to vacuole vesicles with the vacuole or a post-Golgi compartment will have escaped the mutational analysis. Recent evidence confirms that some vacuolar protein precursors are indeed accumulated in post-Golgi transport vesicles and are not secreted (Klionsky *et al.*, 1992).

Second, ten-fold overproduction of CPY results in secretion of the protein via the late secretory pathway (Rothman *et al.*, 1986). The *PRC1* gene, together with other vacuolar proteinase genes, undergoes cell cycle specific regulation and is induced ten to 15 fold in the late logarithmic growth phase (see Chapter 4). Since the standard missorting assay uses cells of early to mid log phase, CPY has been considered as a vacuolar enzyme because it is not secreted under such conditions. This does not mean that CPY is not naturally secreted under certain growth conditions. Thus the isolation of mutants which secrete CPY might also include mutants in hypothetical regulatory paths that regulate *PRC1* gene expression. A way to distinguish such mutants from genuine missorting mutants would be to determine the levels of mRNA and protein synthesis.

Taken together the *vps* mutants isolated so far are not necessarily representative protein sorting mutants, but more likely affect specific pathways that are required for sorting of such proteins in the Golgi or for vesicle budding from the Golgi (class C) mutants and for the regulation of protein expression. Using other vacuolar targeting information from different vacuolar proteins will aid the identification of other genes responsible for specific sorting.

New strategies have to be developed to isolate mutants in the Golgi to vacuole pathway not relying on a *vps* phenotype, but on the absence of a vacuolar compartment for example, assuming that vesicular flow from the Golgi ensures vacuole maintenance. The *ssv* mutants isolated in this study comprise a new set of putative vacuolar mutants, but four complementation groups also have a severe *vps* phenotype. The fact that there is little overlap between *ssv* and *vps* mutants and that some *ssv* mutants have an aberrant vacuole morphology but are able to correctly localize most of their CPY to the vacuole might be characteristic for mutants in a later sorting or targeting pathway. On the other hand, a vacuolar acidification defect induced by specific inhibitors of the vacuolar ATPase or by deletion of vacuolar ATPase genes results in the partial secretion of

vacuolar enzyme precursors (Klionsky *et al.*, 1992; Rothman *et al.*, 1989). This would argue against vectorial transport of vacuolar proteins from the Golgi to the vacuole, assuming that acidic environment in the Golgi is mainatined. Assuming also the presence of intermediate transport vesicles between the distal Golgi and the vacuole these results can be explained by the vesicles fusing back to the Golgi compartment if the vacuole is not acidic, or that these intermediate vesicles fuse with secretory vesicles. Preliminary experimental evidence argues against this model, because acidification deficient mutants have vacuoles and can sort vacuolar proteins to the vacuole, albeit less efficiently. One possible explanation is that vesicles destined for the vacuole fuse with an intermediate pre-vacuolar compartment where sorting receptor recycling takes place. The acidic environment might aid the release of the proteins from a sorting receptor, and if this compartment is not acidic the protein-receptor complex might relocalize to the Golgi where it disassociates and is secreted because of a putative oversaturation of the sorting pathway because most vacuolar proteins are transported back to the sorting compartment. In this context it appears attractive to propose the presence of a pre-vacuolar endosomal compartment acidified by the vacuolar type ATPase because if the recycling took place in the vacuole, the proteins might be subject to proteolytic cleavage. One way to address this hypothesis would be to engineer a C-terminal extention peptide onto CPY which contains a cleavage consensus for PrA. The expression of this peptide in a pulse chase labelling experiment in an acidification deficient vacuolar ATPase mutant and subsequent immunoprecipitation would allow the determination of whether this peptide is cleaved judging from a change in electrophoretic mobility. If the secreted fusion has not cleaved the extention peptide it would favour the argument that the recycling takes place in a pre-vacuolar compartment. On the other hand, Klionsky *et al.*, 1992, suggest that Pho8p and also p2CPY and p2PrA accumulate in vesicles which are unable to fuse with the vacuole. This suggests a completely different role for vacuolar acidification. These results could be explained that for fusion of post-Golgi transport vesicles with the vacuole one of the requirements is an acidic compartment, and if not present the vesicles might, less efficiently, fuse with another acidic compartment - the secretory vesicles.

8.3. Structural Integrity of the Vacuole

Lysosomes in mammalian cells are subject to a reorganization induced by changes in pH (Heuser, 1989). A lowering of the extracellular pH is accompanied by a movement of lysosomes to the periphery of the cell in a microtubule dependent fashion. An increase in pH is accompanied by a movement of the lysosomes to the cell center, both processes wich involve probably kinesin and dynein based motors. Changes in pH also change the fission/fusion properties of lysosomes or lysosomal vesicles. pH dependent changes of vacuolar morphology in yeast have so far not been observed possibly because of the difficulties associated with light microscopic analysis of live vacuoles. However, other pH dependent processes have been described, such as the acidification induced mislocalization of vacuolar proteins (Klionsky *et al.*, 1992). The effect of other environmental changes on the yeast vacuole have not yet been assessed, and it is possible that vacuolar morphology is subject to for example metabolic regulation. Studies in ATP depleted mammalian cells have shown that otherwise separate Golgi compartments can connect by tubules (Donaldson *et al.*, 1989). This is indicative of a malfunctioning of a process involving mechanisms to separate membrane compartments including vesicle budding and fusion. Similarly, the yeast vacuole might undergo morphological changes in mutants if the cytoplasmic environment is unfavourable to the stable assembly of vacuoles and finally disrupt or vesicularize vacuoles, although the mutation itself does not inactivate a vacuolar gene.

8.4. Epilogue

One final question remains - why are there different sorting mechanisms for different vacuolar proteins and different import mechanisms and what is the biological sense of it? Why does the cell require different sorting mechanisms for proteins with the same destination and similar function. The synthesis of proteins involved in sorting and targeting is energetically expensive as is translocation and transport. Most vacuolar proteases are synthesized under energetically unfavourable conditions like starvation or sporulation, and the expense of energy might be unfavourable. The presence of a single

sorting mechanism would help to "save energy". On the other hand strains with several independent mechanisms for vacuolar protein sorting have a selective advantage if one sorting component is inactivated. Also, they might be able to modulate individual transport and sorting activities in response to environmental stimuli.

The biogenesis of the vacuole has been studied by a number of different approaches, mainly initiated by the isolation of mutants deficient in a vacuolar function. Unfortunately, the isolation and characterization of the gene products involved in the biogenesis of the vacuole has not yet allowed us to fully and unambiguously describe the pathway(s) and processes involved in describing the sorting and transport processes. One of the prime targets for future research is to isolate the transport compartment that mediates transport from the Golgi to the vacuole and to prove or disprove that the transport is mediated by intermediate transport vesicles. The sole fact that transport within the secretory pathway is mediated by vesicular intermediates does not necessitate that this is also the case for Golgi to vacuole transport. By analogy, the pulsating vacuole in *Paramecium* sp. derives its contents from morphologically distinctive tubules surrounding it (M. Latterich, unpublished observations). The fact that most fusion processes observed so far are mediated by the NSF (Sec18p), but the vacuolar transport processes do not depend on NSF are intriguing and indicate that this transport pathway might be different to the membrane flow in the secretory pathway. Also not known is the pathway the vacuolar membrane derives its components from. Under normal circumstancs each daughter bud receives some vacuolar material in a regulated fashion, and generally it is believed that the vacuole will grow due to the fusion of transport vesicles or endocytic vesicles with the pre-formed organelle. If this is the case, class C vacuolar mutants should only be deficient in the transport of intermediate vesicles from Golgi to vacuole, whereas all class A mutants are chiefly deficient in the sorting process. The design of a cell free *in vitro* transport reaction reconstituting the step from Golgi to vacuole should greatly aid the elucidation of the mechanism and to test the proposed role of the *VPS* gene products in this pathway. The transport pathway has been reconstituted in gently lysed sphaeroplasts (Vida *et al.*, 1990). So far every effort to reconstitute transport in all *vps* mutants in the presence of wild type cytoplasm

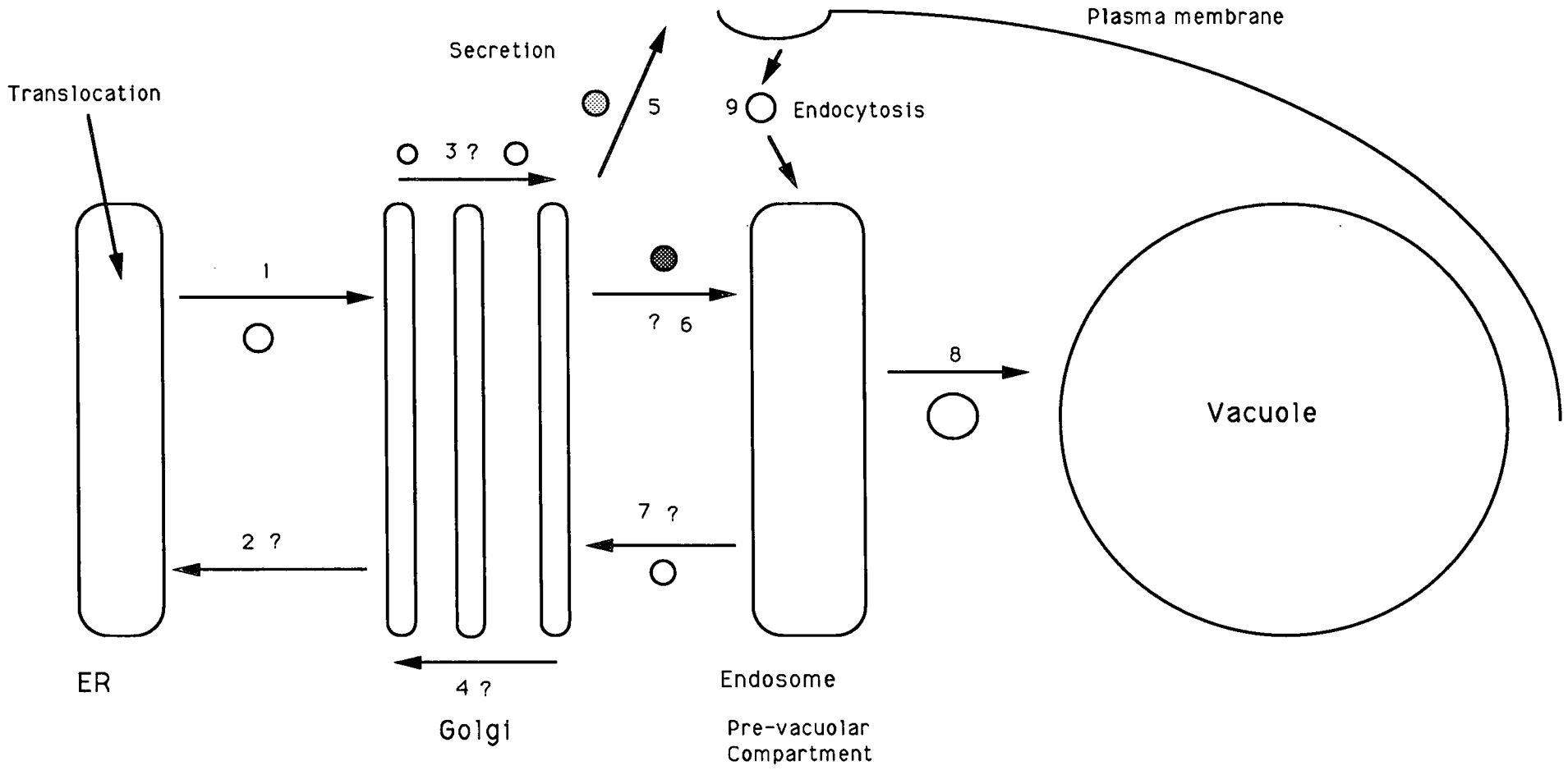
has failed even in *vps* mutants whose gene products are known to be cytoplasmic. This is despite the fact that vacuole segregation in segregation defective mutants could be restored by addition of wild type cytosol in an *in vitro* assay (Tom Vida, personal communication). This is also different to the situation in the secretory pathway where transport can easily be reconstituted *in vitro*. The phenomenon could have several explanations. First, *vps* mutants could have requirements for components which are not sufficiently present under assay conditions. However, transport in the wild type should not occur under the same conditions. Second, all *vps* mutants isolated to date are associated with the Golgi dependent sorting process and/or the process of transport vesicle formation. This would be a good hypothesis for all *VPS* gene products being associated with the Golgi, but it still does not account for soluble *VPS* gene products in the pathway. Third, some of the *VPS* gene products do not participate in transport directly, but are required for the biosynthesis of metabolites which can affect the sorting process. The sole provision of protein factors in the transport reaction might thus not suffice to maintain transport. A provision of intermediate metabolites to the assay might then restore transport activity. Fourth, some *VPS* gene products are inactivated during the cell fractionation process. This does not explain why in wild type broken cells transport does occur. Fifth, the presence of defective *VPS* gene products may not allow wild type *VPS* gene products to become associated with the sorting process. This does not explain why *VPS* deletion mutants still cannot reconstitute *in vitro* transport in the presence of the wild type gene product. Sixth, some *VPS* gene products are involved in the maintenance of a tubular structure spanning from the Golgi to the vacuole. The addition of the wild type gene product will not allow under *in vitro* conditions the formation of the connective tubule.

As a result of the data obtained in this study together with results from other studies describing aspects of vacuole biogenesis, I wish to propose two different models which account for all major findings described up to date. These models are in no case complete, nor do they reflect the *in vivo* situation in yeast vacuole biogenesis. They are merely intended to provide some stimulus for future investigations into vacuole biogenesis from a differnt perspective, and to be disproven rather than proven.

However, they represent alternative models to the existing simple model where Golgi to vacuole transport occurs via vesicular intermediates, and when the sorting process is hindered secretion occurs.

Model A - Vesicular Transport Intermediates. According to this model the transport from Golgi to vacuole is mediated by transport vesicles which bud off from a distal Golgi compartment and fuse with a pre-vacuolar compartment (PVC) which may or may not be identical to an endosomal compartment. This compartment is acidified, and acidification is required to disassociate receptor ligand complexes, before receptors are retrieved in a retrograde vesicle mediated mechanism. The PVC will bud off vesicles which fuse with the vacuole. This model makes biological "sense" in that it allows receptor recycling to occur from a compartment that has no active proteinases present, unlike the vacuole which could "leak" proteinases back to the Golgi if it was the recycling compartment. This model is very attractive in that it is consistent with all experimental findings. If deregulated by the absence of components required for vesicle budding from the PVC, vacuolar proteins would accumulate in this compartment and will eventually be transported to the Golgi via the retrograde transport mechanism and will be secreted because of over-saturation of the sorting process. This would be the case in all class C mutants which do not have a vacuole. The model also accounts for the sorting defects of class A and class B vacuolar mutants, because these mutants do have vacuoles, and the vacuolar material seems to be derived from the secretory pathway by lipid flow which is independent of the sorting of soluble vacuolar proteins, because four mutants (class C) defective in vacuolar protein sorting but not endocytosis do not produce vacuoles (*end1*). One could argue that all but four *VPS* gene products are involved in conferring specificity to the sorting process and are defective in receptors, or related receptor-retrieval processes. One alternative explanation is that transport-intermediate vesicles in the absence of certain gene products can bypass the vacuole and fuse with the plasma membrane. On the other hand, a significant number of *VPS* genes may be involved in intermediate metabolism which when deregulated results in aberrant sorting or a bypass of the vacuole.

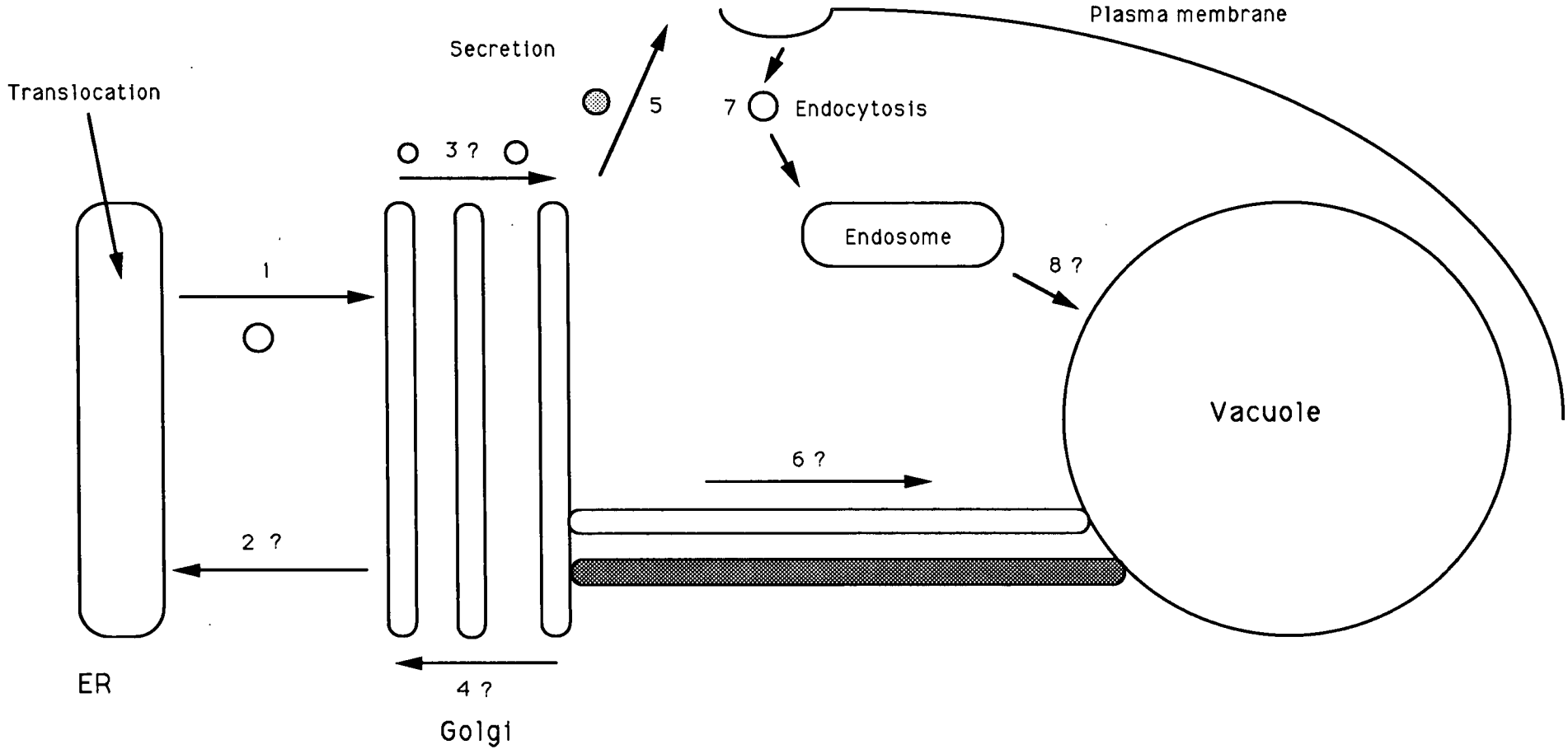
Figure 8.1: Model "A". Transport in *S. cerevisiae* is mediated by vesicles. EDVs bud off from the ER and fuse with the Golgi (1) where recycling of ER proteins takes place via a yet unknown route (2). Intra Golgi traffic in anterograde (3) and retrograde (4) direction is mediated by vesicles. In the *trans* Golgi secretory proteins leave the Golgi by default (5), and vacuolar proteins are retrieved by specific receptors and are transported to the PVC via vesicles (6) which recycle back to the Golgi after dissociation of ligands (7). The PVC will bud off vesicles to the vacuole (8) contributing to vacuolar biosynthesis. Endocytotic vesicles (9) might fuse with the PVC also.


Plasma membrane
Secretion
Translocation
5
9
Endocytosis
3 ?
1
? 6
8
Vacuole
7 ?
2 ?
4 ?
ER
Endosome
Golgi
Pre-vacuolar
Compartment

Model B - Tubular Golgi to Vacuole Intermediate. This model assumes that there are no vesicular intermediates between Golgi and the vacuole, but that a late Golgi compartment is connected to the vacuole via a tubular network. This model is attractive in that it explains the secretion of all soluble vacuolar proteins when Golgi to vacuole transport is disturbed, which would be impossible with intermediate transport vesicles assuming the unidirectionality and specificity of vesicle mediated transport as in the secretory pathway. This model explains why there is no requirement for NSF in this particular pathway. On the other hand the fact that receptor mediated retrieval of vacuolar proteins from the Golgi does occur requires a more sophisticated explanation. It can be imagined that specific receptors transport their ligands along the tubule to the vacuole. If disturbed by changes in pH or mutations that affect the transport within the tubular compartment soluble proteins are secreted, while membrane bound vacuolar proteins will accumulate in the tubule. This model requires the presence of a molecular sieve or "valve" to stop vacuolar contents leaking into Golgi compartments. A morphological analysis of some *vps1* and *ssv1* alleles reveal the presence of "drop" shaped compartments surrounding the central vacuole, similar to the cisternae of a pulsating vacuole in *Paramecium* sp., which might reflect overexaggerated Golgi to vacuole tubules.

Experiments directly addressing aspects of the two models, together with the development of better fixation protocols that preserve ultra-morphological features of *S. cerevisiae* better than existing methods, will lead to more insight in this exciting mechanism of organelle biogenesis. A detailed study of the proposed regulation of vacuolar structure and biogenesis by metabolic intermediates will shed some more light on the integrative regulation of compartmentalized biochemical pathways, and lastly on the mechanism of regulation of organelle structure and function.

Figure 8.2: Model "B". Transport from Golgi to the vacuole is mediated by tubules. EDVs bud off from the ER and fuse with the Golgi (1) where recycling of ER proteins takes place via a yet unknown route (2). Intra Golgi traffic in anterograde (3) and retrograde (4) direction is mediated by vesicles. In the *trans* Golgi secretory proteins leave the Golgi by default (5), and vacuolar proteins are sorted into protein specific tubules which are in direct connection with the vacuole (6). Endocytotic vesicles (7) fuse with an endosomal compartment which subsequently is engulfed by the vacuole or fuses with it (8). A similar process could mediate the uptake of Golgi to vacuole tubules or vesicles in model "A".


Plasma membrane
Secretion
Translocation
5
7
Endocytosis
3 ?
Endosome
1
8 ?
Vacuole
6 ?
2 ?
ER
4 ?
Golgi

Also, an improved method to isolate vacuoles from yeast is desirable because the use of density gradient flotation methods is a lengthy process which easily can inactivate a number of vacuolar constituents (M.D. Watson, unpublished findings). The identification of a vacuolar membrane protein that assembles on the vacuolar membrane and does not take the secretory pathway could help to develop a scheme to affinity purify vacuoles with magnetic beads and thus have a purer and newer preparation.

Finally, "nature" is more creative than the scientific mind of investigators of vacuolar structure and function, who already have difficulties in collecting all relevant information and establishing a link between vacuole structure and function. In different organisms different vacuole-like compartments can have different functions. Apart from *Paramecium* sp. whose pulsating vacuoles take over the role of the "kidney" organelle by collecting and exocytosing water that entered the protozoa during pino- and endocytosis, vacuoles in *Hydractinia symbiolongicarpus* regulate the peristaltic movement of the digestive tract (stolon) in a timely manner by swelling and subsequent contraction to maintain gastrovascular flow (Schierwater *et al.*, 1992): a diverse and sophisticated role for a morphologically "simple" single-layer membrane enclosed compartment.

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