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**CLONING AND CHARACTERISATION OF SSV GENES FROM
*SACCHAROMYCES CEREVISIAE***

BY

CAROLINE ANNE HARTLEY

**A thesis submitted to the Department of Biological Sciences
University of Durham
In accordance with the requirements for the degree of
Master of Science**

March 1995



13 NOV 1995

For the Hartley family

DECLARATION

I declare that the work contained within this thesis submitted by me for the degree of Masters of Science is my own original work, except where otherwise stated, and has not been submitted previously for a degree at this or any other University.

Caroline Hartley

Cloning and Characterisation of *SSV* genes from *Saccharomyces cerevisiae*.

Caroline Anne Hartley

MSc 1995

Abstract.

In order to investigate the pleiotropic effects exhibited by mutations of *S. cerevisiae* defective in genes involved in vacuole biogenesis, a number of mutants were chosen for further study. Three mutants which had been isolated for salt sensitivity (*ssv* mutants) also exhibited defects in a number of unrelated functions including protein targeting and vacuole inheritance. Attempts were made to clone the corresponding wild type genes defined by these mutations.

Plasmid pMLY38 was thought to confer an advantageous growth effect on the *ssv7-1* mutant strain, complementing the salt sensitivity and enhancing its growth on YPDA media supplemented with 1.5M salt. Further analysis of growth curves indicated that pMLY38 did not enhance the growth of *ssv7-1*. Subsequent sequencing indicated that pMLY38 contained the *SSV7* gene. However, an open reading frame (*ORF1*) was detected downstream of *SSV7* and the sequence was analysed. A gene disruption of *ORF1* indicated that it was not an essential gene. Computer analysis of the DNA sequence showed that it encoded a 310 amino acid protein which has a highly acidic C-terminal end. Searches of the data bases showed limited homology to SCYKL202W1 and Nucleolar Transcription Factor 1, the significance of which is unknown.

Characterisation of *ssv16* strains indicated cells with class B vacuoles. Immunoprecipitation results showed the mutants also secrete large quantities of CPY. They also display a semi-dominant phenotype for CPY mis-sorting. In the mutant/wild type diploid CPY continues to be secreted indicating that the mutant phenotype is not completely suppressed by the wild type gene. Cloning of *SSV16* isolated the previously sequenced gene, *SISI*. A study of the growth curves, secretion of CPY and chromosome mapping of *SSV16* failed to ascertain whether *SISI* is identical to *SSV16*.

Immunofluorescence of the *ssv17-101* strains indicated that it belongs to a small group of mutants defective in vacuole segregation. Four transformation methods were employed in order to clone *SSV17* via complementation from a gene bank. Difficulties encountered with these methods resulted in a chromosome mapping technique being employed to locate the gene. This placed the *SSV17* gene on chromosome XIV. Attempts to narrow down the position on the chromosome by meiotic mapping were unsuccessful. Information that a *VAC1* homologue was present on chromosome XIV led to its cloning by PCR in order to determine if it complemented the *ssv17-101* mutation.

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ABBREVIATIONS

| | |
|--------|---|
| bp | base pairs |
| BSA | bovine serum albumin |
| Can | canavanine |
| CDCFDA | 5(6) -carboxy -2',7' -dichlorofluorescein diacetate |
| CTP | cytosine triphosphate |
| CPY | carboxypeptidase Y |
| Da | daltons |
| DAPI | 4',6 -diamino -2- phenylinodole |
| dNTP | deoxyribonucleoside triphosphate |
| DPAP A | dipeptidyl aminopeptidase A |
| DPAP B | dipeptidyl aminopeptidase B |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| FITC | fluorescein isothiocyanate |
| OD | optical density |
| PEG | polyethylene glycol |
| PrA | proteinase A |
| PrB | proteinase B |
| RNAase | ribonuclease |
| SDS | sodium dodecyl sulphate |
| TEMED | <i>N, N, N', N'</i> -tetramethylethylenediamine |
| Tris | tris (hydroxymethyl) aminomethane |
| UV | ultraviolet |
| YGSC | yeast genetic stock centre |

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CHAPTER 1

INTRODUCTION

1.1. General Introduction.

The distinguishing feature of eukaryotic cells is their compartmental organisation of many cellular processes into distinct membrane bound organelles. The unique structural and functional nature of these compartments is conferred by the composition of each organelle by distinct sets of proteins. In order to maintain the identity of each organelle an accurate sorting and trafficking of proteins from their site of synthesis to their correct cellular destination must exist. Therefore much interest has been shown in unravelling this fundamental process of protein sorting and targeting. This process must be highly specific and requires specialised targeting mechanisms and signals. Early work focused on the relationship between the various organelles and a pathway through these organelles was constructed (Rothman and Orci, 1992; Schekman, 1982). This has been followed by the ever advancing study into the molecular machinery involved within each step of the pathway. This work has shown that many components of the secretory pathway are universal, with the same machinery operating within yeast and animals.

The budding yeast, *Saccharomyces cerevisiae*, has become central to the study of protein targeting as they are simple eukaryotes which are relatively easily genetically and biochemically manipulated. The vacuole in yeast shares many features with lysosomes in animal cells and vacuoles in plant cells and has recently become the focus of attention. It is an acidic compartment which contains a variety of hydrolytic enzymes. It is involved in a wide variety of cellular functions: proteolytic processing and degradation of vacuolar and cytoplasmic proteins, amino acid, carbohydrate and ion storage, sporulation and ion- and osmohomeostasis (Klionsky et al., 1990; Raymond et al., 1992).

This chapter will initially review the protein targeting pathway from the endoplasmic reticulum to secretion covering the general features of the organelles involved, the pathway taken, the cellular components that have been characterised and the proposed mechanisms for transport and targeting. A more detailed study of the vacuole will follow, its functions and the processing and targeting of specific vacuolar proteins.

1.2. Secretory Pathway.

The well defined secretory pathway in yeast was characterised by the isolation of conditionally lethal mutations in which protein transport is arrested intracellularly (Novick et al., 1980). These mutants were termed *sec*, for secretory mutants, and fall into two groups. Class A accumulate active secretory enzymes in an intracellular pool, they also accumulate or exaggerate specific secretory organelles (Novick et al., 1980). Class B mutants produce enzymatically inactive forms of invertase and acid phosphatase. Two of these mutants *sec53* and *sec59*, the invertase formed appears to remain embedded in the endoplasmic reticulum (ER) membrane (Schekman, 1982). By simply producing double mutants the order of events within the secretory pathway could be defined. Such double mutants were made by combining, pairwise, each of the four stages identified by the *sec* mutations. These double mutants then accumulate the intermediate prior to the first block encountered. Strains containing *sec53* or *sec59* together with any other mutation fail to accumulate active invertase, making this step epistatic to all the others. The order of events was therefore defined as: entry into the ER; ER to Golgi transport; then secretion or alternatively transport to the vacuole (Novick et al., 1981).

An independent line of inquiry followed the glycosylation of invertase in the *sec* strains. Glycosylation of invertase involves at least two stages. Invertase produced in the *sec53* strain has little or no carbohydrate. On transport through the ER it gains core glycosylation which is then further modified in the Golgi. Various *sec* mutants accumulate different glycosylation intermediates of invertase which confirmed the proposed transport pathway (Esmon et al., 1981).

1.2.1. The Endoplasmic Reticulum.

The start for proteins entering the secretory pathway is passage into the endoplasmic reticulum (ER) through its membrane. Protein translocation into the ER can be divided into two steps; the first is the identification and targeting of the secretory protein and the second is the passage of the nascent protein into or across the ER membrane (Ng and Walter, 1994).

The first step is initiated by the translation of a mRNA encoding a secretory protein. On emergence of the protein signal peptide, binding of the Signal Recognition Particle (SRP) occurs. This signal peptide is usually an N-terminal hydrophobic region. In mammalian cells the functional efficiency of this peptide is related to its hydrophobicity (Bird et al., 1990). Experiments in yeast show that the pre-sequence specificity appears to be low, as a significant amount of random sequences can act as a secretory pathway routing signal (Kaiser and Botstein, 1990).

The interaction of the SRP with the ribosome causes translation of the protein to slow down. This inhibition is released on the binding of the SRP/ribosome/nascent protein complex to the ER membrane, through interaction between the SRP and its membrane receptor (Rapoport, 1990). Following GTP hydrolysis SRP is released and the nascent protein is co-translationally transported through the translocon (a pore in the membrane), and either integrated into the membrane or released into the ER lumen.

The SRP orchestrates the initial targeting of the protein. In mammalian cells it has been shown to comprise a molecule of 7S RNA and 6 polypeptide subunits (relative molecular masses: 9, 14, 19, 54, 68 and 72), which form a cytosolic ribonucleoprotein complex (Siegel and Walter, 1988). The yeast SRP is a 16S particle, of which only three subunits have been identified: two protein units Srp54p, Sec65p and an RNA molecule ScR1 (Hann and Walter, 1991). The mammalian Srp54 is involved in the binding of the signal peptide possibly by the formation of a hydrophobic pocket (Bernstein et al., 1989). It also contains a GTP-binding domain. The complex of the SRP/GTP/SRP receptor, allows freeing of the nascent protein chain from the SRP, and hydrolysis of the GTP releases the SRP back into the cytosol (Connolly and Gilmore, 1993; Connolly et al., 1991). The yeast *SEC65* gene has been characterised and this has significant sequence similarity to another mammalian SRP subunit, *SRP19* (Stirling and Hewitt, 1992). Sec65p is required for the stable association of Srp54p within the SRP (Hann et al., 1992).

Not all of the proteins are targeted by the SRP system. Work with translocation deficient mutants identified three membrane proteins associated with an SRP independent pathway (Deshaies et al., 1991). These membrane proteins Sec61p, Sec62p and Sec63p have been found to form a complex. Coprecipitation experiments show that Sec62p and Sec63p exist as a complex with two additional proteins, 23kDa and the recently characterised 31.5kDa (encoded by the *SEC66/SEC71* gene (Feldheim et al., 1993)) (Deshaies et al., 1991). The association of Sec61p may only be a transient association as in coprecipitation experiments only a few of the total number of molecules have been found in the complex. It has been suggested that the Sec61p-Sec62p complex may act as the signal recognition (Deshaies et al., 1991). In addition, heat shock proteins are also involved: Ssa1p, Ssa2p and BiP (encoded by *KAR2*). A new powerful tool of reconstituted proteoliposomes allows some dissection of the translocation process and this has been used to demonstrate that a complex containing the ER luminal chaperone protein BiP, Sec63p, Sec71p and p23 is required for post-translational translocation (Brodsky and Schekman, 1993).

The *SEC11* gene product and at least one glycoprotein act as the yeast signal peptidase. This complex catalyses the removal of the signal sequence upon or immediately after translocation (Bohni et al., 1988). Once inside the ER the proteins are subject to a number of glycosylation reactions. N-linked glycosylation occurs on proteins exhibiting the Asn-X-Thr/Ser (X= any amino acid except Pro.) which are modified by the addition of

a complex oligosaccharide to the Asn group. Another important modification is O-linked glycosylation by the addition of an N-acetylglucosamine residue to Ser or Thr (Abeijon and Hirschberg, 1992).

Proteins entering the ER are either destined for secretion or targeting to other organelles, those proteins which are destined to remain within the ER must contain a retention signal (reviewed in Nilsson and Warren, 1994). Experiments have shown that a four amino acid sequence, HDEL, is sufficient to retain luminal ER proteins (Pelham, 1989). Judged by their Golgi specific carbohydrate modifications, these proteins leave the ER before being retrieved from an early Golgi compartment (Dean and Pelham, 1990). A sorting receptor *ERD2* was identified (Semenza et al., 1990), which localises to the cis side of the Golgi stack and on ligand binding redistributes to the ER. Ligand binding occurs in a pH dependent manner. Thus, sorting of ER proteins may be aided by pH differences between compartments (Wilson et al., 1993).

1.2.2. Vesicular Mediated Transport

Transport between different organelles and between different compartments of organelles is controlled by vesicular movement (Rothman and Orci, 1992). This vesicular transport allows a convenient method to exchange proteins between distinct membrane compartments. The process can be divided into three convenient steps: vesicle budding, vesicle targeting and vesicle fusion. These processes have been the focus of a substantial amount of work leading to the isolation of many of the components involved. These include coatomers and ARF protein in vesicle budding (reviewed in Kreis and Pepperkok, 1994), and the more recently described SNAP and SNARE mechanism for targeting and fusion of vesicles (reviewed in Takizawa and Malhotra, 1993).

Vesicle movement was initially studied in isolated Golgi stacks by following the transport coupled glycosylation of Vesicular Stomatitis Virus (VSV) G protein. In vitro incubation of the Golgi with cytosol and ATP resulted in the production of vesicles (Balch et al., 1994). Using Golgi prepared from VSV-infected cells the transport between cisterna could be studied by following the glycosylation events of the transported VSV G protein (Balch et al., 1984).

The involvement of coatomers in this process was first identified in experiments using chemical reagents that blocked transport. On addition of these reagents two types of vesicles were seen by electron microscopy (EM). One set of vesicles were enshrouded in an electron dense material. This lacked the well-defined geometry of the clathrin coat, and were referred to as non-clathrin-coated vesicles. The second type were uncoated (Orci et al., 1986). Two chemicals used to study transport blockage were GTP- γ -S and N-ethylmaleimide (NEM). On addition of GTP- γ -S, coated vesicles accumulated and

addition of NEM resulted in the accumulation of uncoated vesicles. Upon combination of both chemicals only coated could be seen, demonstrating the coated vesicles preceded the uncoated variety (Orci et al., 1989).

Purification of Golgi-derived coated vesicles after treatment with GTP- γ -S allowed the characterisation of the coat proteins (COPs). These include α -COP (160kDa), β -COP (110kDa), γ -COP (98kDa), δ -COP (61kDa). The first COP to be identified was the β -COP isolated from rat liver. Its structure showed homology to the β -adaptin of the clathrin coat (Duden et al., 1991) thus suggesting a related role in membrane traffic between COP and clathrin-coated vesicles. More recently ϵ - and ζ -COP have been cloned and sequenced. ζ -COP (20kDa) shows homology to the small subunits of the clathrin-adaptor complexes and is required for the assembly of COP-coated vesicles (Kuge et al., 1993). Characterisation of the ϵ -COP (36kDa) indicated that the coatomer subunit of the COP complex assembles into the coat en-bloc (Hara-Kuge et al., 1994). Electron microscopy work, involving the formation of coated vesicles in vitro when Golgi membranes were incubated with cytosol, backed up the role of coatomer in vesicle formation. Depletion of the coatomer proteins from the cytosol led to the inhibition of vesicle formation which could be reversed by addition of purified coatomer (Orci et al., 1993).

Another protein identified from the coatomer was a Ras-like GTP-binding protein previously characterised as an ADP-ribosylating factor (ARF). ARF exists in two states: cytosolic when bound to GDP and in its active GTP-bound state it associates with the Golgi membrane (Donaldson and Klausner, 1994). ARF functions to drive the assembly and disassembly of the coatomers on the Golgi membrane in a GTP-dependent manner (Donaldson et al., 1992). Work with the fungal metabolite brefeldin A (BFA) supported the idea that coatomer are involved in vesicle formation. Whereas GTP- γ -S promotes coat assembly, BFA inhibits ARF activation and coat association resulting in inhibition of secretion and disassembly and redistribution of the Golgi complex to the ER (Klausner et al., 1992). Although BFA prevented vesicle budding and fusion in a forward direction transport could still occur in a retrograde direction via tubular connections between Golgi stacks (Orci et al., 1991). Subsequently BFA has been shown to inhibit the exchange of GDP for GTP on ARF therefore preventing its association with Golgi membranes and thus preventing coatomer association and vesicle formation. More recent work has focused on the identification of ARF regulators. Evidence for perhaps a trimeric G-protein interaction have been noted including enhanced ARF effects with the specific G-protein activator aluminium fluoride (Donaldson and Klausner, 1994) and inhibition of ARF association to Golgi membrane by the addition of $\beta\gamma$ subunits of G-proteins (Donaldson et al., 1991).

Work on the *sec* mutants of yeast, involved in ER to Golgi transport, provide complementary information in the dissection of vesicle trafficking. These mutants were divided into two classes: class I show few vesicles (*sec12*, *sec13*, *sec16*, *sec23*) whereas class II exhibit an increase in vesicle accumulation (*sec17*, *sec18*, *sec22*). Double mutants

show class I phenotype, therefore class I is epistatic to class II. This result suggests that class I mutants exhibit a defect in vesicle formation, and class II show a defect in vesicle fusion (Takizawa and Malhotra, 1993). Various *SEC* genes have been characterised and show homology to the mammalian coatamer proteins. This suggests a conserved mechanism of non-clathrin-coated vesicle formation (Rothman and Orci, 1992). Sar1p is a Ras-like GTP-binding protein which like ARF exhibits a GTP dependent association with ER membranes. Sec12p is a GDP/GTP exchange factor located in the ER membrane which promotes Sar1p association to the ER membrane (Barlow and Schekman, 1993). Sec23p a GAP with specificity for Sar1p associates with Sec24p (Yoshihisa et al., 1993). Three other proteins are also involved Sec13p, Sec16p and a protein of 150kDa. Addition of these proteins to ER membranes is sufficient to promote the production of fusion competent transport vesicles without the addition of coatamer proteins (Salama et al., 1993). This complicates the proposed role of the coatamer, which had been previously found to be required for vesicle budding. This discrepancy may be due to the differences in the systems studied; ER to Golgi in yeast or Golgi to Golgi in mammals. However *SEC21* has been shown to encode a protein component of yeast coatamer and is required for ER to Golgi transport demonstrating that there may also be a role for COPs. Microinjection of antibodies to β -COP in mammalian cells inhibits vesicle transport, therefore enforcing the suggested role of the coatamer in vesicle trafficking (Pepperkok et al., 1993).

Recent work has indicated that COPs may be involved in other processes such as sorting of cargo into the vesicles. During transport, VSV-G is sorted and concentrated during export from the ER (Balch et al., 1994). It has also been shown that coatamer interacts with di-lysine ER retention motifs suggesting a potential role in retrieval of proteins to the ER (Kreis and Pepperkok, 1994).

The other question asked was how specific vesicles were targeted and fused to their intended membrane. Much insight into this pathway has also been elucidated and a mechanism is beginning to emerge. The first step was the isolation of a protein which could reverse the inhibiting effect of NEM (N-ethylmaleimide) on intra-Golgi transport. This protein was called NSF for NEM sensitive factor and resides as a homotetramer (Ferro-Novick and Jahn, 1994). It has two ATP-binding sites which on mutation of either site results in elimination of the fusion activity promoted by NSF. NSF was found to associate with three SNAPs (soluble NSF Accessory Proteins) in order to bind to the Golgi membrane (Whiteheart et al., 1993). NSF and SNAPs form a complex which can be dissociated by the addition of Mg-ATP. This complex was linked to a column and using brain membranes as the source, membrane bound SNAP receptors, called SNAREs, were isolated (Sollner et al., 1993). Three proteins were identified which were found to be associated with the synapse. One of the proteins found was synaptobrevin which is found

on the surface of the synaptic vesicles, and the other was syntaxin which associates with the pre-synaptic plasma membrane (Sollner et al., 1993). This result immediately suggested a simple method for vesicle docking in which the NSF/SNAP complex links the synaptobrevin in the vesicle to the syntaxin in the membrane. As NSF and SNAPs are general components of the fusion machinery, it appears that the SNAREs are members of a family of proteins which are specific to either the transport vesicle (v-SNARE) or the target membrane (t-SNARE). Different v- and t-SNARE partners could explain the docking specificity at each of the vesicle-mediated steps in eukaryotic cells (Warren, 1993). A third protein isolated by binding to SNAPs is a previously discovered membrane protein SNAP-25. It associates with the t-SNARE.

Some of these genes involved in vesicle transport have been found within yeast substantiating the idea of a conserved vesicle docking mechanism. These proteins have been isolated from mutants defective in protein trafficking. Two *sec* mutants which led to the accumulation of ER-vesicles, *sec17* and *sec18* were found to encode the yeast equivalent of α -SNAP and NSF respectively (Griff et al., 1992).

Five genes related to synaptobrevin have been isolated from yeast: *BOS1*, *BET2*, *SEC22*, *SNC1* and *SNC2*. *BOS1* is involved in protein transport from ER to Golgi and is found within the transport vesicles. Antibodies to Bos1p inhibit the fusion of vesicles to the membrane but do not inhibit budding. Bos1p is not found on Golgi membranes therefore it must either be degraded or recycled after vesicle fusion. *BOS1* also interacts with *BET1* and *SEC22* (Ferro-Novick and Jahn, 1994). *SNC1* and *SNC2* act within Golgi to plasma membrane transport. Both products are required for secretion as yeast strains lacking either *SNC1* or *SNC2* have no apparent phenotype, strains lacking both have a defect in post-Golgi secretion (Protopopov et al., 1993). Sec9p may be the SNAP-25 equivalent in Golgi to plasma membrane secretion as it has some sequence identity (Novick et al., 1980). Related proteins to SNAP-25 in other parts of vesicle transport have yet to be found. Proteins related to syntaxins have also been isolated: Sso1p, Sso2p, Sed5p and Pep12p. Sso1p and Sso2p are found on the plasma membrane the disruption of both genes leads to a block in post-Golgi transport and is lethal. Sed5p is required for ER to Golgi transport and is found on the Golgi apparatus. Pep12p is a protein required for vacuolar sorting.

Control and regulation of the vesicle fusion process has recently been attributed to a large group of proteins known as Rabs (Novick and Brennwald, 1993; Pfeffer, 1994). These are GTPases which inter convert between GDP- and GTP-bound conformations promoting membrane fusion in their GTP-bound form. In yeast two Rabs have been characterised, Ypt1p and Sec4p. Ypt1p works within ER to Golgi transport and Sec4p functions in post-Golgi transport. The current model is that after the fusion of vesicles, the associated GTP-bound form is converted to the GDP-bound form by Rab-specific GTPase activating proteins (GAPs). The inactive Rab is recycled back to the membrane of origin

by a cytosolic protein termed GDI (guanine-nucleotide dissociation inhibitor) (Novick and Brennwald, 1993).

1.2.3. Golgi Apparatus.

The apparent director of post-translational processing and sorting of proteins is the Golgi apparatus. This is an organelle consisting of distinct cisterna arranged in a stack of variable number (Rothman, 1985). The stack is also associated with an array of vesicles and a network of tubules. In mammals much work has been done on the Golgi stack as it can be easily visualised by electron microscopy (EM) and it can be isolated by cell fractionation. The stack has traditionally been thought to consist of four distinct compartments which are involved in the acceptance of proteins from the ER, their subsequent processing and the sorting of these proteins to their final destination. These four subcompartments are termed the cis, medial, trans and trans Golgi network (TGN) (Dunphy and Rothman, 1985). These four stacks are thought to be biochemically distinct. Work following the glycosylation of proteins through the transport pathway and immunolocalisation of the different enzymes involved in these glycosylation processes by electron microscopy helped to distinguish between the various compartments (Rothman, 1985).

Recently the view of a four compartment Golgi has been questioned, with a basic three compartment model being proposed consisting of a CGN (cis-Golgi network), Golgi stacks and TGN (Mellman and Simons, 1992). The CGN and TGN would sort the proteins, and the processing would only occur in the stacks. Residence of the glycotransferases and glycosidases have been shown to vary within the stacks and between different cell types suggesting that Golgi compartmental organisation is less rigid than previously assumed (Valesco et al., 1993). This three compartment model is also consistent with the model proposed for yeast. Yeast Golgi cannot easily be visualised in wild type cells. Isolation of the *sec* temperature-conditional secretion mutants have helped to identify the Golgi stack. *sec7* and *sec14* mutants block post-Golgi protein transport which causes the accumulation of cisterna (Novick et al., 1980). The use of antibodies against Sec7p and Kex2p has allowed the immunolocalisation of the Golgi apparatus in yeast (Franzuoff and Schekman, 1989). A three compartment Golgi has been suggested through work following the events of vacuolar protein sorting in *sec18* mutants (Graham and Emr, 1991).

The CGN is the compartment that acts as the recipient for ER-derived transport vesicles. From here proteins progress through the Golgi stacks or are recycled back to the ER. The medial Golgi functions to mediate glycosylation before handing on to the TGN.

The TGN is the sorting centre which sends the proteins to their correct post-Golgi destination.

Movement between Golgi-stacks has been attributed to vesicular budding and fusion which has been extensively studied (see previous section). However experiments with the inhibitor brefeldin A (BFA) has proposed that intra-Golgi trafficking may occur through transient tubular connections (Orci et al., 1991).

Work on finding Golgi retention or retrieval signals has focused on the Golgi glycosylation enzymes which all have type II topology and are anchored into the membrane by their uncleaved signal peptide. Reporter molecules were shown to localise to the Golgi apparatus if fused to the transmembrane domain and some flanking sequences of various Golgi-resident enzymes, showing that the transmembrane domain was sufficient to act as a retention signal (Machamer, 1993). The mechanism for Golgi retention could not be saturated by overexpression indicating that retention is not a receptor-mediated event. Therefore the transmembrane domain must be a retention rather than retrieval signal (Nilsson and Warren, 1994). Two methods have been proposed. The first involves the formation of long hetero-oligomers between resident proteins which would be prevented from entering the budding vesicle. This oligomer could contain different molecules that are retained in the same subcompartment and could be stabilised by interactions on the luminal or cytoplasmic side of the membrane (Nilsson et al., 1993). The second is retention through membrane thickness. It has been observed that the length and hydrophobicity of the transmembrane domain is important for Golgi retention (Maisbay et al., 1993). Replacement of the transmembrane domain of sialyltransferase with 17 leucines does not prevent its retention in the Golgi, however if it is replaced by 23 leucine molecules the protein will locate to the plasma membrane (Munro, 1991). Observations that the plasma membrane has a high cholesterol level which favours a thicker bilayer suggests that the thickness of the bilayer determines the point at which resident Golgi enzymes are retained (Bretscher and Munro, 1993). There is insufficient evidence at present to corroborate either model.

Within yeast, retention of dipeptidyl aminopeptidase (DPAP) A has been studied. This type II integral membrane protein which is located in the late Golgi was shown to be retained by a 118-amino acid cytoplasmic domain. This domain has further been studied by mutational analysis which has produced a motif, Phe-X-Phe-X-Asp, which is essential for its efficient retention (Nothwehr et al., 1993). Overexpression leads to delivery of the protein to the vacuolar membrane (Roberts et al., 1992) suggesting the existence of a saturable retention binding site.

A role for Vps1p in Golgi membrane protein retrieval has been described (Wilsbach and Payne, 1993). *VPS1* encodes a 704 amino acid hydrophilic protein with a predicted molecular weight of 79kDa (Rothman et al., 1990). Vps1p is comprised of two functional domains. It has the ability to bind and hydrolyse GTP, and this amino-terminal domain

shares identity with other GTP-binding proteins including the vertebrate Mx proteins, the *Drosophila melanogaster shibire* proteins, the yeast *MGM1* protein and bovine brain dynamin (Vater et al., 1992). The resident Golgi protein *KEX2* has been studied and the cytoplasmic domain has been shown to act as the retention sequence. *lam1* mutants were identified which mislocalise Kex2p to the vacuole. *LAM1* is allelic to *VPS1* (Wilsbach and Payne, 1993). It is proposed that Kex2p travels to an intermediate endosome-like compartment before being recycled back to the late Golgi. The pathway from endosome back to the Golgi is blocked in the *vps1* mutant (Wilsbach and Payne, 1993). The question left unanswered is whether both these proposed retention or retrieval systems play a part in Golgi enzyme localisation

1.2.4. Secretion.

Secretion is the default pathway for soluble proteins entering the transport pathway in mammalian, yeast and plant cells (Denecke et al., 1990). Proteins transit the ER to the Golgi where they are sorted and targeted to specific locations. Any soluble proteins which contain no further targeting information enter the default pathway which results in secretion (Burgess and Kelly, 1987). Experimental evidence supports this pathway. Work with glycopeptides indicates that transport to the cell surface occurs by default. This glycopeptide shows a very rapid rate of secretion which is faster than other secretory proteins taking about 10 minutes to transit the secretion pathway (Weiland et al., 1987). This suggested that no special structural motif or signals are necessary for rapid and efficient transport from the lumen of the ER to the cell surface. Isolation of signal sequences for some intracellular proteins has strengthened the role of secretion as the default pathway. Additional supportive evidence is shown by alteration in the ER retention signal, KDEL, results in protein secretion (Munro and Pelham, 1987) and removal of the vacuolar sorting signal leads to missorting of CPY to the cell surface (Valls et al., 1987). Overproduction of CPY leads to its secretion (Stevens et al., 1986) which is blocked in secretory mutants where a late step in constitutive secretion is inhibited.

Although secretion is a constitutive process it is correlated topologically to the region of cell growth within yeast cells. It involves polarised deposition of new cell surface material at the bud site (Schekman, 1982).

1.3. Vacuoles.

The vacuole is a prominent organelle in yeast occupying roughly 10-20% of the cell volume (Raymond et al., 1992). It appears as a complex, multi-lobed structure which

during cell division is partitioned to the daughter cell ensuring inheritance and indicating a specific mechanism for vacuole segregation (Weisman et al., 1987).

The vacuole undertakes a variety of overlapping, often related functions in concert. It is an acidic compartment with the pH maintained at 6.0 by the vacuole H⁺ATPase, which also provides the correct conditions for the efficient activity of the hydrolytic enzymes. These are resident in the vacuole and function in proteolytic processing and intracellular macromolecular degradation. The electrochemical gradient developed by proton pumping drives the import of several metabolites across the vacuole membrane. The vacuole plays an important role in the storage of various metabolites, the removal of various inhibitory ions and the regulation of those ions involved in regulatory or signalling pathways (reviewed in Klionsky et al., 1990). It serves as a major storage reserve for basic amino acids, carbohydrates, inorganic phosphates and calcium ions and these reserves are mobilised in response to nutrient limitations such as nitrogen starvation. Another function of the organelle is osmoregulation, a process which is little understood and which is discussed in section 1.6..

The features of the vacuole make it similar to both mammalian lysosomes and plant vacuoles (Raymond et al., 1992). Due to the facile nature of yeast genetics and biochemistry the roles and functions of the vacuole has become an important area of study.

1.3.1. Vacuole Inheritance.

During budding the daughter bud acquires a vacuole, the volume of which increases in size as the bud grows (Weisman et al., 1987). Cytological studies have shown the daughter cell vacuole is not entirely formed from newly synthesised material but shows inheritance of maternal vacuolar material (Weisman et al., 1987; Weisman et al., 1990). This transfer is restricted to a path between the mother and daughter cells and is co-ordinated with the cell cycle (Gomes de Mesquita et al., 1991; Weisman et al., 1990).

The vacuole in the mother cell projects tubulovesicular structures to the bud, termed segregation structures, which delivers vacuolar material to establish the daughter vacuole early in S phase (Weisman and Wickner, 1988). This process is completed by the onset of nuclear migration to the bud neck that precedes nuclear division (Raymond et al., 1990, M.D. Watson unpublished observation). These vesicular structures fuse within the daughter bud which leads to mixing of the vacuolar content and formation of a new vacuole (Weisman et al., 1990).

The process of vacuole inheritance has been reconstituted *in vitro* using isolated vacuoles (Conradt et al., 1992), this has allowed dissection of the *in vitro* reaction into four distinct sequential biochemical subreactions (Conradt et al., 1994). Stage I requires vacuoles in a moderately ionic solution, this may serve to prepare the vacuole membrane

to allow subsequent binding of cytosolic factors. Stage II involves Stage I vacuoles and cytosol, during this stage cytosolic factors required for the targeting and/or fusion of segregation structures may assemble into pre-fusion complexes on the vacuolar membrane. ATP is required for Stage III which initiates vacuole to vacuole fusion. Actual fusion of the vacuoles does not occur until Stage IV which appears to be dependent on the concentration of vacuoles. Inhibitor susceptibility has led to further characterisation of the pathway (Conradt et al., 1994). Prior to completion of Stage III the reaction is susceptible to inhibitors of the ATPase such as bafilomycin A₁ and the proton ionophore CCCP, this suggests an electrochemical potential across the vacuole membrane is important for fusion initiation (Stage III) but not at the actual membrane fusion event. Inhibition by mastoparan and GTPγS also at this stage suggests the involvement of GTP-hydrolysing proteins (Haas et al., 1994). A protein dephosphorylation step may be necessary for fusion as it has been shown that Step IV is blocked by inhibitors of protein phosphatases type 1 and 2A.

1.3.2. Synthesis and Processing Pathway of Vacuolar Proteins.

The intensive study of the vacuolar glycoprotein carboxypeptidase Y (CPY) has provided information about its synthesis and transportation to the vacuole. CPY is synthesised as an inactive preproenzyme (67kDa) which is converted into an active mature form of 61kDa involving two proteolytic steps (Hasilik and Tanner, 1978). Using temperature sensitive secretory mutants (*sec*) the compartmental processing of CPY was discerned (Stevens et al., 1982). *sec* mutants interrupt transport in the secretory pathway at different stages at the non-permissive growth temperature (37°C), this may result in the accumulation of the ER or Golgi bodies it also results in accumulation of the precursor forms of CPY. The pathway involves production of the preproprotein and its translocation into the ER, where the signal peptide is cleaved by a signal peptidase (Blachly-Dyson and Stevens, 1987). Two forms of CPY were found by immunoprecipitation of pulse-labelled CPY after accumulation of protein in mutant cells. p1CPY (67kDa), accumulated in *sec18-1* cells, and represented the ER modified form of CPY. In *sec7* and *sec14* mutants, which accumulate Golgi stacks, a 69kDa precursor was identified and designated p2CPY. This represents the fully glycosylated CPY precursor. Secretory vesicle mutants such as *sec1* accumulated the mature CPY (61kDa) at the restrictive temperature. *pep4* mutants accumulated the enzymatically inactive form of proCPY. *PEP4* encodes proteinase A which removes the prosequence within the vacuole (Mechler et al., 1987). Double mutants made between *pep4* and either *sec18*, *sec7* or *sec1* helped to define the maturation process of CPY. *sec18* and *sec7* were epistatic to the *pep4* mutation and mainly produced p1CPY at 37°C. In *pep4* or *pep4 sec1* double mutant the p2CPY form accumulated, showing the late secretory pathway is not involved in CPY maturation.

The difference in mass between p1 and p2CPY are attributed to the carbohydrate side chains. Endo H treatment of both forms produces deglycosylated polypeptides of the same size (Stevens et al., 1982). During transport through the secretory pathway CPY undergoes core-glycosylation modifications on four Asn-X-Thr acceptor sites in the ER. This produces the p1 form. Outer chain modification occurs in the Golgi to produce the p2 form (Esmon et al., 1984).

Parallel to this work experiments were undertaken using fusions of CPY sequences peptide to reporter genes. This elucidated the peptide sequence that targeted the proprotein to the vacuole. In mammals mannose-6-phosphate serves as the secondary signal that targets proteins to the lysosome (Kornfeld and Mellman, 1989). Experimental evidence suggested that this mechanism was not used in yeast. CPY and other vacuolar enzymes can be targeted to the vacuole in the presence of tunicamycin, a drug that blocks the addition of N-linked oligosaccharides (Stevens et al., 1982). Site directed mutagenesis eliminating all four glycosylation acceptor sites present in CPY did not lead to missorting of the protein product, although it remained unglycosylated (Winther et al., 1991).

Fusion of amino terminal segments of CPY to the reporter enzyme invertase showed that the N-terminal 50 amino acids of CPY are sufficient to direct the delivery of a CPY-Invertase hybrid protein to the yeast vacuole (Johnson et al., 1987). This 50 amino acid segment contains two distinct functional domains: a 20 amino acid N-terminal signal peptide followed by a 30 amino acid vacuolar sorting signal. Mutational analysis of the propeptide region of the CPY protein indicated that 4 amino acid residues near the N-terminus are necessary and sufficient for the functioning of the CPY vacuolar localisation signal. These four amino acids are QPRL and are positioned at amino acids 24 to 27 of preproCPY (Valls et al., 1990).

1.4. Mutants Defective for Vacuolar Biogenesis.

The isolation of mutants with defects in protein transport have greatly helped the elucidation of the mechanism by which this process occurs. Isolation of the *sec* mutants in *S. cerevisiae*, which were incapable of protein secretion under specific conditions (Novick et al., 1980), revealed groups of genes that encode products required for transport of proteins between various secretory pathway organelles. Similarly as the isolation of *sec* mutants has helped to increase the knowledge of the mechanisms involved in early protein targeting and transport, isolation of mutants involved in vacuole biogenesis are beginning to clarify the later stages of protein targeting. A number of mutational approaches have been taken to examine aspects of vacuole biogenesis. Surprisingly most genes involved in one aspect of vacuole biogenesis are also defective in other related or unrelated aspects, this leads to an extensive overlap of the genetic complementation groups identified

(Raymond et al., 1992). It also prevents the unambiguous identification of whether a defect is primary or secondary. The following sections describe some of the different approaches taken to identify vacuole mutants.

1.4.1. Isolation and Characterisation of Vacuolar Protein Sorting Mutants.

The default pathway for soluble proteins is secretion which occurs to proteins if they enter the ER with no other retention or targeting information. Based on this, mutants were isolated according to their mislocalisation of CPY to the cell surface. Two main isolation methods using this information as a basis for selection were devised (Bankaitis et al., 1986; Rothman and Stevens, 1986).

One method relies on the fact that a small amount of mislocalised CPY, which is secreted as the Golgi-modified proCPY precursor, is proteolytically activated by an extracellular protease (Rothman and Stevens, 1986). Active CPY is able to catalyse the peptide bond cleavage of the N-blocked dipeptide N-CBZ-L-Phe-L-Leu. Leucine auxotrophs are able to maintain growth on medium containing N-CBZ-L-Phe-L-Leu as the sole leucine source if active CPY is available. Leucine auxotrophs carrying a *pep4-3* mutation are unable to grow on this medium as vacuolar CPY cannot be matured to its active catalytic form. Mutants of the *pep4 leu2* haploids that secrete significant amounts of p2CPY as a result of a vacuole mislocalisation defect are able to grow on this media as a result of maturation of the CPY by the extracellular protease, thus providing a selection procedure. Eight *VPL* (for vacuolar protein localisation) complementation groups were identified in the initial report (Rothman and Stevens, 1986).

The second method relied on the fact that extracellular invertase is required for utilisation of sucrose as a carbon source. This procedure takes advantage of the fact that a CPY-Invertase fusion is sequestered in the vacuole, therefore if present in a Suc⁻ strain the invertase is not available to utilise the sucrose medium. If the sorting process is affected by a mutation then the gene fusion is secreted and such mutants are able to grow on sucrose medium. This procedure has been used to isolate 33 complementation groups termed *VPT* for vacuolar protein targeting mutants (Bankaitis et al., 1986; Robinson et al., 1988). Complementation analysis between both sets of mutants showed an extensive genetic overlap and led to a consolidation of nomenclature and renaming to vacuolar protein sorting or *VPS* genes (Robinson et al., 1988; Rothman et al., 1989).

The *vps* mutants all share certain similarities. They secrete 20-95% of Golgi modified proCPY. The fact that they all secrete the p2CPY precursor is indicative of a sorting defect immediately following the Golgi dependent modification of CPY, before it reaches the vacuole. Most of the mutants tested also secrete several other vacuolar hydrolases, namely PrA and PrB (Robinson et al., 1988) suggesting that the *vps* mutants

influence a common sorting mechanism. Construction of *vps sec1-1* double mutants showed accumulation rather than secretion of p2CPY at 37°C, indicating that sorting occurs at a stage in the Golgi apparatus prior to secretory vesicle formation and that mislocalised p2CPY is secreted via the normal late secretory pathway (Rothman and Stevens, 1986). Of the *vps* mutants tested there was no detectable presence on the cell surface of vacuolar membrane proteins showing that the sorting of soluble and membrane bound vacuolar proteins are independent.

Various methods have been used to visualise the vacuole using light and electron microscopy (Banta et al., 1988). Initial characterisation of the *vpt* mutants indicated that there were at least three classes of vacuolar morphology (Banta et al., 1988). Class A mutants had large vacuoles, class B had a multiply fragmented vacuole and class C strains had no apparent vacuoles. Further studies characterising all the *vps* mutants used indirect immunofluorescence with antibodies to the integral membrane protein Pho8p or the 60kDa peripheral membrane subunit of the vacuolar H⁺-ATPase. This work extended the classes to include three new groups (Raymond et al., 1992).

Class A has seven members whose vacuoles are indistinguishable from the wild type they are: *vps2*, *vps8*, *vps12*, *vps13*, *vps30*, *vps32* and *vps44*. The class B mutants: *vps5*, *vps10*, *vps17*, *vps41*, *vps42*, *vps43* and *vps46* have a fragmented vacuole. The four class C mutants: *vps11*, *vps16*, *vps18* and *vps33* appear to have no vacuoles, but accumulate numerous aberrant vesicular bodies. In addition to missorting CPY these strains have many pleiotropic phenotypes associated with them. Class D mutants have a normal vacuole according to the anti-Pho8p fluorescence pattern but show a vacuolar segregation defect. Whereas the maternal cell possesses a vacuole during budding segregation structures are hardly observed and many buds have the appearance of class C mutants. In addition there is no staining using anti-60kDa, and quinacrine in these mutants does not cause any fluorescence. This is consistent with a defect in vacuolar ATPase assembly. *vps3*, *vps6*, *vps9*, *vps15*, *vps19*, *vps21*, *vps26*, *vps29*, *vps34*, *vps35*, *vps38* and *vps45* belong to this group. The largest group of mutants are those belonging to class E: *vps4*, *vps14*, *vps20*, *vps22*, *vps23*, *vps24*, *vps25*, *vps27*, *vps28*, *vps31*, *vps36*, *vps37* and *vps39*. These have a similar vacuole to wild type with anti-Pho8p staining but the 60kDa localises to a non vacuolar compartment. This suggests that the components of the vacuolar ATPase are mislocalised. *vps1* belongs in a group by itself as it consists of a large central vacuole surrounded by smaller vacuolar fragments.

In addition to the vacuolar protein sorting defects exhibited by these mutants, many other functions are affected: vacuolar assembly, organelle acidification, cell growth, sporulation and osmoregulation (Klionsky et al., 1990). This indicates that the vacuole is involved in a number of interdependent functions, which are no longer maintained if a mutation in vacuolar biogenesis occurs. Therefore characterisation of many of the *VPS*

genes may not only help the understanding of the eukaryotic protein sorting pathway but may also help to unravel some of the other functions that the vacuole is involved in. Characterisation of some of the *VPS* genes has already led towards the understanding of CPY sorting. Vps10p is the CPY receptor (Marcusson et al., 1994). Vps15p and Vps34p work together possibly in a signal cascade system which may result in vesicle formation (Stack et al., 1993). Vps1p has been characterised as a GTPase and is thought to be involved in retention of proteins in the Golgi (Vater et al., 1992). Other *vps* mutants have been characterised but their biological functions are still unknown.

1.4.2. Vacuolar Segregation Mutants.

Inheritance of the vacuolar compartment from mother to daughter involves the transport of maternal vacuolar material to the bud. Isolation of mutants that block intervacuole vesicular traffic and vacuole segregation has produced evidence to support the idea that the daughter bud derives its vacuole from vacuolar material provided by vesicular traffic from the mother cell (Weisman et al., 1990). The isolation of these mutants has helped to clear up a contested pathway. These mutants which are defective in vacuolar segregation were isolated by screening *vps*-like mutants for those exhibiting temperature sensitive growth. About 50% of mutants that lack a vacuole are temperature sensitive. Of 23 sensitive mutants 6 had defects in vacuole division and segregation. In these mutants mislocalisation of proteins required for the partitioning of the vacuole during division could be indirectly causing the abnormal vacuole inheritance. Alternatively these mutants may affect proteins which are directly involved in vacuole division and segregation. Two of these mutants have been characterised. *vac1-1* mutants are temperature sensitive, missort CPY to the cell surface and block the normal transfer of the endogenous vacuolar fluorophore between parent and daughter cell. The *vac1-1* inheritance phenotype is the formation of a large bud which fails to inherit vacuolar material from the maternal cell. This defect specifically acts on the vacuole, as the nucleus and mitochondria sort normally to the bud (Weisman et al., 1990). The *VAC1* gene has been cloned and encodes a 515 amino acid protein. This appears to have no homologies in the data bases and the most notable aspect of the sequence is the presence of three zinc fingers (Weisman and Wickner, 1992). Vac1p may be a peripheral membrane protein, or a protein associated with the cytoskeleton (Weisman and Wickner, 1992). Studies of the *vac1-1* mutant reveals that vacuoles can also arise in the bud without significant inheritance from the maternal vacuole, however this is a slower and infrequent process.

The *Vac2-1* mutation exhibits a temperature-sensitive defect in vacuole segregation but does not show any mislocalisation of CPY (Shaw and Wickner, 1991). This indicates that vacuole segregation and protein sorting are two unrelated processes, making *VAC2*

unique in that it may encode a component of the segregation apparatus as opposed to being due to the pleiotropic effect of another vacuolar function. Work using in vitro reactions which reflects the process of vacuole inheritance has established the requirements of *VAC1* and *VAC2* proteins for the production of tubulovesicular structures from vacuoles in vitro or within semi-intact cells (Conradt et al., 1992).

1.4.3. Isolation and Characterisation of Salt-Sensitive Vacuolar Mutants.

Observations that some *vps* mutants display an osmosensitive phenotype led to the proposal of vacuolar function in osmoregulation. Four *vps* mutants: *vps11*, *vps16*, *vps18* and *vps33* lack a vacuolar structure, secrete soluble vacuolar enzymes and are sensitive to high osmotic strength media. Four others have either disrupted vacuoles (*vps1*), or possess aberrant organelles (*vps15*, *vps34*, *vps40*) (Raymond et al., 1992). To study the process of vacuole formation and to help define the involvement of the vacuole in osmoregulation mutants were isolated which had an inability to tolerate high osmotic stress (Latterich and Watson, 1991).

After mutagenization by either EMS (ethylmethanesulphonate) or UV light, 97 independent mutants were isolated by negative selection of replicas on 1.5M NaCl-containing YPDA medium. These 97 strains fail to grow on high osmotic media. Complementation analysis showed that they fall into 17 groups which were termed salt sensitive vacuolar (*ssv*) (Latterich and Watson, 1991). Twenty three mutants only showed weak complementation with a large proportion of the other *ssv* mutants. They are, however, phenotypically very similar and completely fail to complement each other. These were assigned *ssv*-100 to *ssv*-122 and were put in a separate group *SSV17* (Latterich, 1992). Only three groups have been identified before, *SSV1* is allelic to *VPS1*, *SSV9* is allelic to *VPS33* and *SSV15* is identical to *OSM2* (Latterich, 1992).

The complementation groups were classified according to vacuolar morphology using electron and light microscopy. Five groups show class A vacuoles: *ssv4*, *ssv10*, *ssv11*, *ssv12* and *ssv14*. Class B has seven groups: *ssv2*, *ssv5*, *ssv6*, *ssv7*, *ssv8*, *ssv13* and *ssv16*. Class C consists of *ssv1*, *ssv3*, *ssv9*, *ssv15* and *ssv17*. Mutations in each complementation group were tested for secretion of several vacuolar proteinases, CPY, PrA and PrB. Results showed that each group secreted vacuolar proteins to different degrees (Latterich, 1992). These observations and classifications have been subsequently modified by indirect immunofluorescence and further complementation tests [this thesis, M.D. Watson, T.S. Stevens unpublished observations]

Two genes have so far been characterised. *SSV15* is identical to *OSM2/ARO7* which encodes chorismate mutase. This enzyme is involved in aromatic amino acid biosynthesis (Latterich, 1992; Latterich et al., 1993).

SSV7 has been cloned and its sequence reveals a 926 amino acid protein that contains two conserved domains found in ubiquitin processing proteases (Latterich et al.,). The stress sensitivity of *ssv7* mutations is in accord with other components of the ubiquitin pathway which have been shown to be involved in other stress responses such as resistance to heat shock, starvation and resistance to Cd²⁺ ions. The mild *vps* phenotype exhibited by *ssv7* strains suggests a link between vacuole biogenesis and ubiquitin metabolism (Latterich et al.,).

The wide variety of genes identified which have a role in vacuole biogenesis suggest that vacuolar development is extremely plastic in that a variety of unrelated defects can have pleiotropic effects on vacuolar functions (Latterich et al., 1993).

1.5. A Current Model for the Sorting of Vacuolar Proteins.

1.5.1. Soluble Proteins.

Efficient sorting of soluble vacuolar proteins requires the presence of cis-acting sorting information to segregate them from the bulk of the secretory proteins headed to the cell surface. These targeting peptides provide the information to cause their sequestration in the vacuole but the process by which this occurs is still under investigation. Isolation of vacuolar (*vps*) mutants have produced more than 40 complementation groups implicating a large number of genes are involved in the process of sorting. Many *vps* mutants exhibit extreme defects in the localisation of soluble vacuolar hydrolases while alkaline phosphatase (ALP) localisation appears to be normal (Raymond et al., 1992). Overproduction of CPY causes it to be missorted to the cell surface suggesting a receptor mediated pathway (Stevens et al., 1986), however this does not cause secretion of other soluble vacuolar enzymes implying that each vacuolar protein has a separate receptor or sorting pathway (Paravicini et al., 1992). However, some components of the sorting machinery must be shared because some of the *vps* mutants mislocalise both CPY and proteinase A (PrA).

Candidates for shared components include *VPS15* and *VPS34*. Mutants in these genes exhibit severe defects of the sorting of vacuolar hydrolases. Essentially all of the CPY is mislocalised as the p2 form to the cell surface. However, the vacuole of these mutants is near-normal and they display only a modest defect in the maturation of the membrane protein alkaline phosphatase (Banta et al., 1988). These observations indicate that *VPS15* and *VPS34* are required for the specific sorting of soluble vacuolar enzymes.

VPS15 encodes a 1455 amino acid protein whose 300 amino acid N-terminal end exhibits significant homology to the catalytic domains of the serine/threonine-specific

family of protein kinases (reviewed in Herman et al., 1992). Alterations within the conserved region of the kinase domain of Vps15p results in a temperature sensitive growth defect, missorting and secretion of vacuolar proteins and biological inactivation of *VPS15* (Herman et al., 1991). Vps15p is a phosphoprotein. In vivo this suggests that it catalyses a specific autophosphorylation reaction. It is associated with the cytoplasmic face of the Golgi or vesicles. *vps15 Δ C30* encodes a truncated *VPS15* that lacks the C-terminal 30 amino acids. This exhibits severe temperature sensitive defects in delivery of CPY to the vacuole at the non-permissive temperature. However, delivery and maturation of the membrane protein ALP is unaffected. The *vps15* kinase-domain mutants also exhibit specific defects in Vps15p phosphorylation (Herman et al., 1992).

The *VPS34* gene product shares significant sequence similarity with the catalytic subunit of mammalian phosphatidylinositol (PI) 3-kinase (Schu et al., 1993). PI 3-kinase phosphorylates PI and other more highly phosphorylated PI derivatives (PIP and PIP₂) at the D-3 position of the inositol ring. In mammalian cells PI 3-kinase associates with many signal-transducing receptor tyrosine kinases and is thought to be involved in the key second messenger molecules important in regulating cell growth and proliferation. *S. cerevisiae* has PI 3-kinase activity and strains deleted for *VPS34* lack this activity. Point mutations within the conserved regions of *VPS34* result in decreased PI 3-kinase activity and a severe sorting defect. Elevated levels of PI 3-kinase activity can be detected in overexpression of *VPS34* (Schu et al., 1993), this suggests that the gene encodes a PI 3-kinase and that it is involved in the regulation of protein trafficking.

Evidence has also been shown for a direct interaction between Vps34p and Vps15p. Overproduction of Vps34p in a *vps15* kinase domain point mutation strain suppresses both the growth defect and mislocalisation of vacuolar proteins. However, overproduction cannot suppress the *vps15* null allele (Stack et al., 1993). This suggests a functional interaction between the two proteins. Native immunoprecipitation and chemical cross-linking experiments demonstrate that Vps15p and Vps34p are physically associated and this complex has been located to the late Golgi (Stack et al., 1993). Point mutations in the *VPS15* kinase domain result in defective PI 3-kinase activity suggesting that a Vps15p mediated phosphorylation event is required for activation of the *VPS34* PI 3-kinase (Stack et al., 1993).

A model has been suggested for the activity of Vps15p and Vps34p in soluble vacuole protein sorting (Herman et al., 1992). This proposes that ligand binding to vacuolar protein receptors may transduce a signal that promotes the activity of Vps15 protein kinase. A Vps15p-mediated protein phosphorylation event could activate Vps34p which would phosphorylate membrane PI this could have the effect of physically stimulating membrane curvature or could be linked to a cascade of events resulting in vesicle formation. This has Vps15p and Vps34p acting as components of a membrane-

associated signal transduction complex that couples the binding of the soluble protein by a receptor molecule to a second messenger molecule that could trigger the action of effector proteins.

A role for clathrin in vacuolar protein sorting has also been proposed. Temperature sensitive mutants of clathrin heavy chain (*chc1-ts*) display a severe defect in soluble vacuolar protein sorting at the non-permissive temperature, however, targeting of ALP is unaffected. Incubation for extended lengths of time at the non-permissive temperature results in the restoration of protein sorting. Deletion of *CHC1* shows no sorting defect. These results show that although clathrin may play a role in protein sorting there is a mechanism which can substitute for it if its function is eliminated (Seeger and Payne, 1992).

Mutations in three genes were found to specifically missort CPY: *VPS10*, *VPS35* and *VPS29*. These appear to have no other sorting defects and were therefore classed as potential sorting receptors for CPY. *VPS10* has been characterised and has been designated as the sorting receptor (Marcusson et al., 1994). The *VPS10* gene encodes a 1577 amino acid type I transmembrane protein. The subcellular fractionation pattern of Vps10p closely matches that of the late Golgi marker protein Kex2p locating it to the late Golgi. Chemical cross-linking studies show that Vps10p and the Golgi-modified precursor of CPY directly interact. A single amino acid change in the vacuolar localisation signal QRPL of CPY prevents its association to Vps10p (Marcusson et al., 1994). Hence Vps10p is the receptor for p2CPY. This complex is first transported to the endosome where the p2CPY is released and transported to the vacuole. Vps10p would be recycled to the trans-Golgi compartment. The cytoplasmic tail of Vps10p contains a potential retrieval motif consistent with this theory.

A role for Vps29p and Vps35p has also been indicated due to their specificity for CPY although no mechanism has been proposed. *VPS35* has been cloned and it encodes a protein which is peripherally associated with the cytoplasmic face of Golgi membranes which ruled it out as the CPY receptor.

1.5.2. Membrane Associated Proteins.

The default pathway for integral membrane proteins in mammalian cells is the cell surface along with soluble proteins entering the transport pathway (Denecke et al., 1990). Removal of retention signals from integral membrane proteins of the ER, Golgi and lysosome result in their delivery to the plasma membrane. This was also thought to hold true for the yeast integral membrane proteins. More recently work has been undertaken and results point to targeting of membrane proteins to the vacuole as the default pathway, therefore proteins destined to the vacuole require no further targeting information.

DPAP B (dipeptidyl aminopeptidase B) is a vacuolar integral membrane protein, it is a 120kDa glycoprotein which is encoded by *DAP2*. The amino-terminal 29 amino acids are mainly hydrophilic residues that constitute the cytoplasmic domain, this is followed by 16 hydrophobic membrane spanning amino acids. The remaining 76 amino acids are the luminal part which comprises the enzymic portion (Roberts et al., 1989). Indirect immunofluorescence has located DPAP B to the vacuole in yeast. In order to study any cis-acting targeting signals on DPAP B fusion proteins were constructed between it and the Golgi membrane protein DPAP A or invertase (Roberts et al., 1992).

Fusion of the cytoplasmic and membrane spanning region of DPAP B to invertase resulted in localisation of the protein to the vacuole indicating that the luminal domain was not required for vacuole targeting. Replacement of the membrane spanning domain of DPAP B with that of DPAP A and separately the removal of the cytoplasmic domain of DPAP B also resulted in functional DPAP B localised to the vacuole membrane. Therefore no evidence could be found for a specific vacuole targeting region in DPAP B (Roberts et al., 1992).

Additional support for the default pathway to be the vacuole comes from studying the targeting of the Golgi proteins DPAP A and Kex1p. DPAP A is a 120kDa type II integral membrane protein with similar topology to DPAP B (Roberts et al., 1989) Kex1p is a 110kDa integral membrane protein. Overexpression of these proteins in protease deficient *pep4* yeast strains result in mislocalisation to the vacuole membrane (Cooper and Bussey, 1992; Roberts et al., 1992). Mutations and deletions in the retention domain within the cytoplasmic tail also results in delivery of the proteins to the vacuole (Cooper and Bussey, 1992; Roberts et al., 1992).

Complicating these observations is the result that in a clathrin heavy chain mutant, Kex2p and DPAP A are delivered to the cell surface. This suggests that clathrin has a role in retention of specific Golgi proteins but complicates the notion of the vacuole as the default pathway (Seeger and Payne, 1992).

1.6 Osmoregulation.

The osmotic stress response is the term given to the molecular events provoked by the exposure of yeast cells to high osmotic strengths (reviewed in Mager and Varela, 1993). A decrease in the external water potential results in the outflow of water molecules from the cells into the medium. A shift to high salt affects several essential cellular functions such as nutrient uptake and protein biosynthesis. The cells also have to cope with a general increase in internal solute concentrations caused by cell shrinkage, an influx of toxic solutes and loss of cytoskeletal components.

In order to understand the pleiotropic effects of osmostress the processes involved in the stress response have been extensively studied. The acquisition of osmotolerance relies on the accumulation of osmolytes inside the cell to counteract the elevation of the external osmolarity. ^{13}C nuclear magnetic resonance spectroscopy has shown glycerol to be the major osmolyte, although arabinol and trehalases have also been found (Reed et al., 1987).

Cells can acquire osmotolerance if they are pre-treated in 0.7M NaCl. During this conditioning the rate of glycerol formation increases along with the rate of its secretion therefore maintaining intra- and extracellular glycerol concentration (Edgley and Brown, 1983). The activities of GPDH (glycerol phosphate dehydrogenase) and phosphofructokinase are also elevated in cells subjected to osmotic stress (Edgley and Brown, 1983). However, glycerol is not the sole protector against osmostress. Glycerol accumulation in cycloheximide treated cells did not protect against osmotic shock indicating that protein synthesis is also required to establish the osmotic state (Blomberg and Adler, 1989).

Isolation of mutants by various methods may help to define the action of osmotolerance. The vacuole has been shown to be involved in osmohomeostasis as mutants in vacuolar function have also been shown to be sensitive to high salt concentrations (Banta et al., 1988; Latterich and Watson, 1991). The mutants may have altered vacuolar structure and all secrete soluble vacuolar proteins. Studies by Latterich and Watson (Latterich and Watson, 1993) may indicate that the flow of water from the vacuole into the cytoplasm may act as an immediate response to increased external osmolarities before accumulation of glycerol takes over, which lags behind the initial exposure by several hours (Singh and Norton, 1991).

Another set of osmosensitive mutants, *act1-1* and *act1-2*, are temperature sensitive and result in a rapid and reversible change in actin filament organisation (Novick and Botstein, 1985). Suppressors have been isolated and there is evidence that one encodes an actin associated protein (Chowdhury et al., 1992).

Recently a two-component signal transduction system has been proposed to mediate the response to high salt concentrations. Initial studies identified genes required for restoring the osmotic gradient across the cell membrane in response to an increase in external osmolarity (Brewster et al., 1993). These genes are named *HOG* for high osmolarity glycerol response. *HOG1* is a member of the mitogen-activated protein (MAP) kinase family and *HOG4* was found to be identical to *PBS2* which encodes a putative MAP kinase kinase (MAPKK). Hog1p is tyrosine phosphorylated, by Pbs2p, in response to increased osmolarity and it seems likely that these two proteins function in a signalling pathway enzyme cascade in response to osmotic pressure (Brewster et al., 1993).

The role of signal transduction in response to diverse environmental signals have been extensively studied in bacteria. In prokaryotes the first part of the two component system consists of a histidine kinase which autophosphorylates on a histidine residue

within its catalytic kinase domain in response to environmental stimulus. The phosphate is transferred to a response regulator which then binds an output protein to effect a cellular response. Within yeast the *SLN1* gene was shown to encode a protein which has significant sequence homology to bacterial sensor molecules (Maeda et al., 1994). Sln1p contains both a histidine kinase domain and a receiver domain. It has hydrophobic sequences resembling membrane-spanning domains which suggests it is an integral membrane protein with an extracellular 'sensor' domain. Suppressors of Sln1p were isolated and designated *ssk1* to *ssk4*. *SSK3* was identical to *HOG1* and *SSK4* was identical to *PBS2* suggesting a link between Sln1p and the *HOG* osmosensing MAP kinase related cascade pathway. *SSK1* was shown to encode a protein kinase with a receiver domain homologous to the bacterial response regulator molecules. With analogy to the bacterial system a model for osmolarity response is proposed (Hughes, 1994; Maeda et al., 1994). This is where *SLN1* histidine kinase regulates the phosphorylation state of *SSK1*. Phosphorylated *SSK1* is inactive whereas the unphosphorylated form regulates the *HOG1* MAP kinase pathway. The *HOG* MAP kinase pathway must respond to several inputs as loss of either *HOG1* or *PBS2* function confers sensitivity to high osmolarity whereas loss of *SSK1* and *SLN1* does not.

1.7. Aim of this Study.

In order to study the role of the vacuole in osmohomeostasis salt sensitive vacuolar (*ssv*) mutants were isolated by M. Latterich (Latterich, 1992). Initial characterisation showed that the mutants exhibited defects in a number of unrelated functions. These included vacuolar defects, sensitivity to high osmotic strength compounds and missorting of soluble vacuolar proteins.

This study aimed to clone and sequence the genes corresponding to three of these mutations in order to analyse certain aspects of vacuolar biogenesis at a molecular level.

MLY1517 (*ssv7-1*) is complemented by *pMLY38*, a plasmid isolated from a genomic library. This plasmid was thought to confer an advantageous growth effect on the *ssv7-1* strain. The aim was to isolate and characterise this element and study the interaction, if any, it had with *SSV7*.

The second aim of this study was to clone and characterise two of the *ssv* strains. *ssv16* has a semi-dominant secretion phenotype and *ssv17-101* which belong to a small group of mutants which exhibit a vacuolar segregation defect.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials.

2.1.1. Reagents and Suppliers.

All inorganic chemicals were of AnalaR quality and were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. unless otherwise specified.

Sodium chloride was from Riedel-de Haen, Seelze, Germany.

All organic chemicals and enzymes were from Sigma Chemicals Plc., Poole, Dorset, U.K. unless otherwise specified.

Agar bacteriological (no.1) and yeast extract were from Oxoid Ltd., Basingstoke, Hants., U.K..

Trypticase peptone was from BBL, Cockeysville, U.S.A..

Bacto peptone and Yeast nitrogen base without amino acids were from Difco Laboratories, Detroit, MI 48232-7058, U.S.A..

Restriction endonucleases, corresponding buffers, T4 DNA ligase, Klenow enzyme, X-gal and wild-type λ DNA were from NBL, Cramlington, Northumberland, U.K., Boehringer Mannheim (UK) Ltd., Lewes, U.K., or New England Biolabs, CP Labs Ltd., Bishop's Stortford, Hertfordshire, U.K..

Mung bean deletion kits were from Stratagene, La Jolla, CA92037, U.S.A..

Nylon hybridisation transfer membranes and radiochemicals were from Amersham Ltd., Bury, U.K..

Trans [^{35}S]-label was from ICN Biomedicals Ltd., High Wycombe, Bucks., U.K..

Agarose was from BRL, Gaithersburg, U.S.A..

Fuji RX-100 X-ray film was from Fuji Photo Film Co., Ltd., Japan.

Polaroid film was from Polaroid (UK) Ltd., St. Albans, Hertfordshire, U.K..

Filter paper (3MM) and laboratory sealing film were from Whatman International Ltd., Maidstone, U.K..

Zymolyase 100-T was from Seikagaku Kogyo Co, Ltd., Tokyo, Japan.

Silica fines were provided by Nigel Robinson, Durham University, U.K..

IgG Sorb was from The Enzyme Centre, Malden, MA 02148, U.S.A..

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

Amino Acid Stock Solutions.

Stock solutions of individual amino acids were prepared at 2mg/ml and then autoclaved. They were used at a final concentration of 20 μ g/ml as supplements of yeast minimal medium as required.

Antibiotic Stock Solutions.

Ampicillin was prepared as a 50mg/ml stock solution in 70% ethanol, and used at a final concentration of 50 μ g/ml

6x Agarose Gel Loading Buffer.

| | |
|------------------|------|
| Bromophenol blue | 25mg |
| Xylene cyanol | 25mg |
| Sucrose | 4g |

in 10ml dH₂O. Stored at 4°C

Agarose was from BRL, Gaithersburg, U.S.A..

Fuji RX-100 X-ray film was from Fuji Photo Film Co., Ltd., Japan.

Polaroid film was from Polaroid (UK) Ltd., St. Albans, Hertfordshire, U.K..

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6x Agarose Gel Loading Buffer.

| | |
|------------------|------|
| Bromophenol Blue | 25mg |
| Xylene Cyanol | 25mg |
| Sucrose | 4g |

in 10ml dH₂O. Stored at 4°C

50x TAE Buffer.

| | |
|---------------------|--------|
| Tris | 242g |
| 500mM EDTA (pH 8.0) | 100ml |
| Glacial Acetic Acid | 57.1ml |

in 1L dH₂O.

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

| | |
|-------------------|------|
| 1M Tris.HCl pH8.0 | 10ml |
| 250mM EDTA pH8.0 | 4ml |

in 1L dH₂O.

2.1.3. 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 | 10g |
| Yeast Extract | 5g |
| NaCl | 5g |

in 1L dH₂O.

10x M9 Salts.

| | |
|----------------------------------|------|
| Na ₂ HPO ₄ | 6g |
| KH ₂ PO ₄ | 3g |
| NaCl | 0.5g |
| NH ₄ Cl | 1.0g |

in 100ml dH₂O.

M9 Minimal Medium.

To 1.5% Agar in 90ml dH₂O cooled to 50°C add;

| | |
|----------------------|--------|
| 10x M9 salts | 10ml |
| 1M MgSO ₄ | 0.2ml |
| 20% Glucose | 1ml |
| 1M CaCl ₂ | 0.01ml |

Amino acids and antibiotics were added as required.

Sporulation Agar.

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

in 1L dH₂O. Essential amino acids were added after autoclaving to a final concentration of 20µg/ml.

Yeast Minimal Medium.

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

in 1L dH₂O. Required amino acids were added after autoclaving to a final concentration of 20µg/ml.

YPD(A).

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

in 1L dH₂O.

2.2. Strains and Plasmids.

2.2.1. Bacterial Strains.

The bacterial strain used in this study was *Escherichia coli*, and strains are listed in table 2.1. All strains represent laboratory stocks.

E. coli strains were cultured in Luria broth at 37°C. Cultures of bacteria containing plasmids were grown in media containing the appropriate antibiotic selection. Strains were maintained for short periods of time on L-agar. Stocks of all bacteria were maintained in 15% glycerol at -80°C.

| Strain | Genotype | Reference |
|--------|---|-------------------------|
| DH5a | <i>supE44 ΔlacU169 (ϕ80 lacZΔM15)</i> <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> | (Sambrook et al., 1989) |
| JA221 | <i>recA1 leuB6 trpΔE5 hsdR⁻ hsdM⁺ lac Y</i> | (Beggs, 1978) |

Table 2.1. Bacterial strains used in this study.

2.2.2. Plasmid strains.

All plasmids used in this study were maintained in DH5α. Except the plasmid involved in gene deletion which was maintained in JA221. Plasmids and their most prominent features are listed in table 2.2.

| Strain | Genotype | Reference |
|--------|---|-------------------------------|
| PUCI9 | <i>amp^R</i> | (Yanisch-Perron et al., 1985) |
| YCp50 | <i>tet^R, amp^R, URA3</i> | (Rose et al., 1987) |
| pRS316 | <i>amp^R, URA3</i> | (Sikorski and Hieter, 1989) |
| pL2 | <i>pUC12 LEU2</i> | C. Raymond, Oregon |

Table 2.2. Plasmids used in this study.

2.2.3. Yeast Strains

The yeast strains used in this study are listed in table 2.3. and were all *Saccharomyces cerevisiae*. The mapping strains were all obtained from the Yeast Genetic Stock Center (YGSC). MLY strains and MEY0508-26 are all laboratory strains derived from the parental strains SEY6210 and SEY6211, they are all *leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 suc2-Δ9 GAL*.

All yeast strains were grown with agitation in YPDA or minimal media supplemented with the appropriate amino acids at 30°C except temperature sensitive strains which were maintained at 27°C. Strains were kept for short term storage on agar plates.

Stocks of all yeast strains were kept in 40% glycerol at -80°C

Table 2.3. Yeast strains used in this study.

| Strain | Genotype | Reference |
|------------|--|-------------------------|
| SEY6210 | <i>MATα leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL</i> | (Robinson et al., 1988) |
| SEY6211 | <i>MATa leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL</i> | (Robinson et al., 1988) |
| MLY1511 | <i>MATα ssv7-1 leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL</i> | (Latterich, 1992) |
| MLY1517 | <i>MATa ssv7-1 leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL</i> | (Latterich, 1992) |
| MLY0508 | <i>MATa ssv17-101 leu2-3,112 ura3-Δ52 his3-Δ200 rp1-Δ901 ade2-101 suc2-Δ9 GAL</i> | (Latterich, 1992) |
| MEY0508-26 | <i>MATα ssv17-101 leu2-3,112 ura3-Δ52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL</i> | M. El Hassi |

| Strain | Genotype | Reference |
|------------|--|-----------------------|
| MLY1156-2B | MAT α <i>ssv16-1 leu2-3,112 ura3-Δ52 his3-Δ200</i> <i>trp1-Δ901 lys2-801 suc2-Δ9 GAL</i> | (Latterich, 1992) |
| XS1-3A | MAT α <i>rad52-1 ade1(I) leu2(III) trp1(IV)</i> <i>ura3(V) his2(VI) arg4(VIII) his6(IX) ilv3(X)</i> <i>met14(XI) lys9(XIV)met2(XIV) arg1(XV) gal1</i> | YGSC |
| AB1380 | MATa <i>ade2-1 can1-100 lys2-1 trp1</i> <i>ura3 his5 [ψ^+]</i> | YGSC |
| DBY1710 | MATa <i>kar1-1 ura3-52 tris 3-</i> | (Novick et al., 1989) |
| 368 | t.s. ⁺ MATa <i>rna2-1 ade1 ade2 ura1 his7 lys2</i> <i>tyr1 gal1</i> | YGSC |
| B-7588 | CHRI MATa <i>cir^o ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2</i> | YGSC |
| B-7170 | CHRII MATa <i>cir^o ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7171 | CHRIII MATa <i>cir^o ura3-52 trp1-289</i> <i>his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7589 | CHRIV MATa <i>cir^o ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7590 | CHRV MATa <i>cir^o ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |

| Strain | Genotype | Reference |
|--------|---|-----------|
| B-7591 | CHRVII MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2</i> | YGSC |
| B-7173 | CHRVII MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7174 | CHRVIII MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7175 | CHRIX MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7593 | CHRX MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2</i> | YGSC |
| B-7178 | CHRXI MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7595 | CHRXII MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7255 | CHRXIII MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7596 | CHRXIV MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7180 | CHRXV MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 his3-Δ1 met2 cyh^r</i> | YGSC |
| B-7598 | CHRXVI MATa cir ^o <i>ura3-52 leu2-3,112</i> <i>trp1-289 met2 cyh^r</i> | YGSC |

2.3. General Methods.

2.3.1. Sterile Working Practices.

Sterile working practices were maintained throughout. All media or materials were autoclaved at 15 p.s.i., for 20 minutes, unless heat sensitive, in which case they were filter sterilised by passing through a 0.22µm nitrocellulose membrane filter. Heat sensitive plasticware was sterilised by leaving overnight in 70% ethanol then washing in sterile dH₂O.

All bottle necks were flamed before and after use, and caps were replaced on all containers as soon as possible. Culture plates were sterilised after pouring by flaming the surface of the molten agar medium with a roaring Bunsen flame.

2.3.2. Measurement of Yeast Growth.

Total cell counts of yeast cultures were determined with a Coulter® Multisizer II fitted with a 37µm aperture. The window was adjusted to take account of all particles between 0.748 and 10.00µm. A sample of the yeast culture was diluted 100 times with Isoton® electrolyte solution and two counts were made per sample, subtracting the blank value. The cell number could be calculated as follows:

Total Cell Count/ml = Cell Count (for 200µl) x 40 x Dilution Factor

2.4. Classical Yeast Genetics.

2.4.1. Sporulation of Diploids.

Diploids of the cells to be sporulated were patched onto YPDA agar incubated for two days at 30°C then replica plated onto sporulation agar. Spore plates were incubated at room temperature for 5 days, until tetrads had formed as detected by light microscopy. A loopful of the sporulated diploids (about the size of a match head) were resuspended in 100µl 1:10 dilution of glusulase. The suspension was incubated at room temperature for 8 minutes then 5-10µl were streaked along one side of a YPDA plate. The plate was inverted over the microscope on a specially designed plate holder. Spores were visualised under a

Nikon, Labophot-2 microscope (Nikon, Japan) using a 20x objective lens and 10x eyepieces. The spores were dissected by picking up a tetrad on a needle mounted in a micromanipulator (Narishige Co., Ltd, Tokyo, Japan. Model MO-202). The four spores were separated and plated at 5mm intervals across the plate. The plate was incubated at 30°C for 2-3 days until small colonies appeared. These haploids were picked and tested for various markers.

2.4.2. Chromosome Mapping - via Chromosome Loss (Mortimer and Contopoulou, 1991).

This method relies on the loss of a single specific chromosome in a diploid resulting in expression of the unmarked recessive mutant phenotype. A set of 16 cir^0 tester strains, each have plasmid DNA containing the 2μ inverted repeat sequence integrated at or near the centromere of a different chromosome. The 2μ plasmid DNA remains stably integrated in cir^0 strains since the plasmid DNA lacks the 2μ FLP gene required for site specific recombination. Specific loss of integrated 2μ plasmid DNA and the chromosome into which integration occurred is induced in a cir^0/cir^+ diploid since FLP function provided by the 2μ circles of the cir^+ parent catalyzes a site specific recombination event. This results in the loss of the integrant plus the entire chromosome containing the integrant.

The recessive mutation in a cir^+ strain, was assigned to its chromosome by crossing to each of the cir^0 tester strains and isolating the cir^0/cir^+ diploids on a selective medium. The diploids were grown twice in 10ml YPDA liquid media. Cells were counted and approximately 100 cells plated onto YPDA agar. These cells were replica plated onto a media that selected for the expression of the unmapped mutation. The recessive mutation is only manifested in the diploid containing the cir^0 tester strain in which the integrant is on the same chromosome as the mutation.

2.4.3. Gene Mapping (Sherman and Wakem, 1991).

The unmapped gene was assigned to a location on a chromosome by crossing to various strains carrying mutations which have been mapped to known positions on the chromosome, and determining whether or not linkage exists between the unknown gene and the markers used.

The strain with the unmapped gene and the tester were streaked onto YPDA agar and grown overnight at 30°C. They were replica plated onto YPDA so the strains crossed on the plate and grown overnight. This was then replica plated onto a minimal media plate supplemented only with amino acids that were essential to both strains, leaving out those which are required by only one strain. Only diploids grew, which were sporulated and the

spores separated. Tetrad analysis was used to determine the proximity between the two markers by studying the ditypes and determining the number of crossover events which had occurred between the unknown gene and the marker.

2.5. 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.

2.5.1. Phenol:Chloroform Extraction of DNA.

To remove proteins from DNA solutions an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and the phases mixed. The emulsion was centrifuged at 12,000 g for 30 seconds and the aqueous phase transferred to a fresh tube. The extraction process could be repeated until no precipitate at the interphase was formed. The aqueous phase was transferred to a fresh tube after each extraction. An equal amount of chloroform:isoamylalcohol (24:1) was added to remove traces of phenol, the tube was centrifuged and the aqueous phase, containing the DNA, transferred to a fresh tube. The DNA could then be recovered by ethanol precipitation.

2.5.2. Precipitation of DNA with Ethanol.

0.1 volume of 3M sodium acetate (pH4.8) and 2.5 volumes of 100% ethanol were added to the DNA containing solution, mixed and stored at -80°C for 30 minutes. DNA was pelleted by centrifugation at 12,000 g for 10 minutes. Pellets were washed in 70% ethanol, the supernatant removed and the pellet allowed to dry before being resuspended in TE buffer.

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

The protocol used was that supplied with the Qiagen Plasmid Maxi or Midi kits. Up to 500µg of plasmid DNA could be obtained from a 500ml starting culture using a Qiagen tip 500, or up to 100µg of plasmid DNA from a 50ml starting culture using a Qiagen-tip 100.

2.5.4. Plasmid DNA Preparation from *E. coli* - Silica Fines Preps.

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

5ml Luria broth containing the appropriate antibiotic were inoculated with a single bacterial colony, and incubated at 37°C overnight with vigorous agitation (200 r.p.m.). 1.5ml of the culture was transferred into an eppendorf and centrifuged for 1 minute at 12,000 g in a microcentrifuge. The supernatant was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 350 μ l of STET buffer (8% sucrose, 0.5% (v/v) Triton X-100, 50mM EDTA, 10mM Tris.HCl pH8.0.), 25 μ l of a freshly prepared lysozyme solution (10mg/ml lysozyme in TE buffer) was added, and the tube vortexed for 3 seconds. The tube was incubated in a boiling waterbath for exactly 90 seconds, and immediately centrifuged at room temperature for 10 minutes. The pellet was removed from the tube with a sterile toothpick and 0.7ml NaI solution (90.8g NaI, 1.5g Na₂SO₃ in 100ml dH₂O, filter sterilized, and 0.5g Na₂SO₃ added after filtering) was added to the supernatant. 5 μ l of resuspended, acid washed silica fines (silica 325 mesh, 50% (v/v) in dH₂O) were added to the tube, mixed by inversion, and the plasmid DNA bound to the glass by incubation at room temperature for 30 minutes. The tubes were centrifuged for 15 seconds at 12,000 g and the supernatant removed by aspiration. The pellet was washed in 1ml 70% (v/v) ethanol:30% (v/v) TE buffer, centrifuged and the ethanol removed leaving the pellet as dry as possible. The fines were resuspended in 50 μ l TE buffer and the DNA eluted by incubation 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.

Preparation of silica fines:

250ml of silica 323 mesh powder was resuspended in distilled water to give a total volume of 500ml. The suspension was stirred for 1 hour and left to settle for a further hour. The suspension was then centrifuged at 5000g in a beckman J2-HS centrifuge using a JA-14 rotor. The pellet was resuspended in 150ml of distilled water and 150ml nitric acid. The suspension was then heated to 98°C and allowed to cool to room temperature. The silica fines were then repeatedly washed with sterile water until the pH was greater than 5.5. Silica fines were stored at 4°C as a 50% slurry in sterile distilled water.

2.5.5. Plasmid DNA Preparation from *E.coli* - Alkaline Lysis.

An alkaline lysis method of plasmid preparation with a PEG precipitation step was employed to provide DNA for sequencing.

5ml Luria broth containing appropriate antibiotics were inoculated with a single bacterial colony, and incubated overnight with agitation. 3ml of the culture was centrifuged at 6000 g for 5 minutes. The supernatant was removed by aspiration, leaving the pellet as dry as possible. The pellet was resuspended in 100 μ l of ice cold TGE (25mM Tris.HCl pH8.0, 50mM glucose, 10mM EDTA) and incubated at room temperature for 5 minutes. 200 μ l of a freshly prepared solution containing 0.2M NaOH and 1% SDS was added, the tube mixed by inversion and incubated on ice for 5 minutes. 150 μ l of an ice cold acetate solution (11.5ml glacial acetic, 28.5ml water, 60ml 5M potassium acetate) was added and incubated on ice for a further 5 minutes. The tube was centrifuged for 5 minutes and the supernatant transferred to a fresh tube avoiding any precipitate. RNase A was added to a final concentration of 20 μ g/ml and incubated at 37°C for 20 minutes. An equal volume of phenol:chloroform was added, the tube vortexed then spun to separate the layers. The aqueous phase was transferred to a fresh tube and the DNA precipitated with 2.5 volumes of ethanol at -20°C for 10 minutes. After centrifugation the pellet was washed twice with 200 μ l 70% ethanol, dried briefly and resuspended in 16 μ l dH₂O, 8 μ l 2M NaCl and 20 μ l 13% PEG₈₀₀₀. The contents were thoroughly mixed, then left on ice for 20-30 minutes. The DNA precipitated was collected by centrifugation at 12,000 g for 10 minutes, and washed three times with 200 μ l, 70% ethanol. The pellet was dried briefly before finally resuspending in 20 μ l TE.

2.5.6. Preparation of total Yeast DNA, Limited Purity (Campbell and Duffus, 1988).

Yeast cells were grown in 20ml YPDA to late log phase. 5ml aliquots were harvested, resuspended in 1ml of 1M sorbitol and transferred to an eppendorf tube. The cells were spun in a microcentrifuge and resuspended in SP buffer (1M sorbitol, 50mM potassium phosphate buffer pH7.5). 2 μ l of β -mecaptoethanol was added and the tube incubated at room temperature for 10 minutes. 50 units of Glusulase was added and incubated for 30 minutes at 30°C. Cells were washed in 1M sorbitol then resuspended in 50mM EDTA, pH8.5. 20 μ l of 10% SDS was added, heated for 15 minutes at 70°C and transferred to ice. 100 μ l of cold 5M potassium acetate solution was added, gently mixed and kept on ice for 1 hour. The tube was centrifuged at top speed for 10 minutes in a microcentrifuge the supernatant transferred to a fresh tube and ethanol added with gentle mixing to fill the tube. The tube was spun again for 10 minutes to precipitate the DNA, the ethanol removed and the pellet briefly dried before resuspending in 0.5ml TE. RNaseA

was added at 10 µg/ml and the tube incubated at room temperature for 3 hours before performing three phenol extractions. DNA was obtained by ethanol precipitation and the pellet was resuspended in 50µl of TE.

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

This procedure was adapted from a method provided by Janet Shaw (personal communication). A 10ml overnight of yeast cells in YMM with the appropriate amino acids were grown with agitation to mid log phase. Less than 20 OD units were harvested at 3000 r.p.m for 5 minutes and the pellet resuspended in 1ml PE (50mM potassium phosphate pH7.5, 25mM EDTA). The cells were transferred to an eppendorf tube, centrifuged at 12,000 g for 2 minutes and resuspended in 150µl PE plus 5mM DTT, freshly prepared. Lyticase was added to 50 units for every OD unit harvested, the tube then incubated at 37°C for 40 minutes. 150µl of a solution of 2% SDS, 0.1M Tris.HCl pH9.0, 10mM EDTA, was added, the tubes vortexed and incubated at 65°C for 5 minutes. 150µl 5M potassium acetate was added, the tube vortexed and incubated on ice for 30-60 minutes. This was centrifuged in the cold room at 12,000 g for 5 minutes and the supernatant transferred to a fresh tube. 200µl of 5 M ammonium acetate was added and the tube filled with isopropanol then incubated at -20°C for 10 minutes. Following a 3 minute spin the supernatant was removed and the pellet dissolved in 90µl dH₂O. 10µl 5M ammonium acetate and 200µl isopropanol were added and the contents of the tube mixed before centrifuging for 2 minutes. The pellet was washed in 80% ethanol and dried briefly before resuspending in 50µl TE. The tube was then centrifuged and the supernatant transferred to a new tube.

This preparation produces sufficient plasmid to transform into *E.coli* to amplify. A range of DNA concentrations between 0.5µl and 10µl of the preparation was used as using excess DNA has been observed to inhibit *E.coli* transformation.

2.5.8. Polymerase Chain Reaction (PCR) Amplification.

For a single 100µl PCR reaction the following components were added to a 0.5ml sterile eppendorf tube:

- 1x Taq polymerase buffer
- 160pmoles forward and reverse primers
- 1.5mM MgCl₂
- 500ng genomic DNA
- dH₂O to 79µl final volume.

Tubes were heated to 99°C for 6 minutes then transferred to ice for 1 minute before adding

2.5 units Taq DNA polymerase and 200 μ M dNTP's. The reaction mixture was spun briefly to mix the components prior to the addition of a 100 μ l mineral oil overlay. Amplification was carried out in a thermal cycler (PHC-3, Techne). Denaturation was carried out at 95°C for 1.5 minutes, annealing at 55°C for 1.5 minutes and extension at 72°C for 2 minutes. The PCR cycle was repeated 22 times then a final cycle of 95°C for 1.5 minutes, 55°C for 1.5 minutes and 72°C for 10 minutes. The reaction was then cooled to 4°C. The DNA from the reaction was electrophoresed on an agarose gel and the band isolated as described in section 2.6.12..

2.5.9. Restriction Digests.

Restriction digests were carried out using commercial sources of enzymes and their corresponding buffers, as supplied by the manufacturer. Restriction enzymes were used at 1 unit per μ g of DNA to be digested. Digests were carried out for a minimum of 2 hours at 37°C. Double digests were carried out simultaneously in One Phor All buffer supplied by Pharmacia.

If digestions were to be analysed by gel electrophoresis, 0.2 volumes of 6x gel loading buffer were added. If the digestion was to be used in further subcloning steps, the digestion was stopped by removing the restriction enzyme(s) using Promega "Wizard clean up" spin-columns according to the manufacturers instructions.

2.5.10. DNA Molecular Weight Markers.

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

| | |
|-----------------------------------|-------------|
| λ DNA (300 μ g/ml) | 100 μ l |
| dH ₂ O | 780 μ l |
| 10x Restriction Enzyme Buffer | 100 μ l |
| Restriction Enzyme (10U/ μ l) | 20 μ l |

Digestion was carried out for 7 hours at 37°C. 200 μ l 6x stop dye was added, and the markers stored at -20°C. 15 μ l was loaded onto agarose gels as a size standard.

λ *Pst*I digest produces DNA fragments of the following sizes (in kb):

14.05, 11.49, 5.07, 4.75, 4.51, 2.84, [2.56, 2.46, 2.44], 2.14, 1.99, 1.70, 1.16, 1.09, 0.81, 0.52, 0.47, 0.45, 0.34..

The fragments in brackets run together on an agarose gel.

2.5.11. Agarose Gel Electrophoresis.

Agarose gel electrophoresis was performed in an Appligene mini-gel system. Agarose was added to a measured quantity of 1x TAE buffer, usually to a concentration of 0.7% (w/v), this efficiently separated linear DNA between 10-0.8 kb. The concentration of agarose could be varied according to the size of DNA to be separated (Sambrook et al., 1989). The agarose was dissolved by microwaving the mixture. After cooling, ethidium bromide was added to a final concentration of 0.5 μ g/ml and the agarose poured into the mould with a well comb in place. Once the agarose had set, the gel was placed into the tank and covered with 1x TAE buffer containing 0.5 μ g/ml ethidium bromide. DNA samples in 6x sample buffer were loaded and electrophoresis performed at 80mV 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.

2.5.12. DNA Fragment Isolation.

This method was a personal communication from Dr N. Robinson, and uses the solutions described in section 2.6.4.

DNA fragments separated in an agarose gel were excised with a sterile razor blade and put into a 1.5ml eppendorf, 1ml NaI solution was added and the tube heated at 70°C until the agarose had melted. The tube was allowed to cool then 5 μ l silica fines added, the DNA was bound and eluted from the fines as described previously (section 2.6.4.).

2.5.13. Filling in 3'-Recessed Termini.

The DNA fragment (maximum of 500ng) was resuspended in 10-15 μ l of sterile distilled water following isolation from an agarose gel. A 1 μ l solution containing all 4 dNTPs (each at 1mM) was added to the DNA. 2 μ l of Klenow buffer (10x) was added (supplied with the enzyme) and the reaction buffer made up to 20 μ l with sterile distilled water plus 1 μ l (1 unit) of Klenow fragment. The reaction mixture was left at room temperature for 30 minutes and then the Klenow fragment inactivated by incubating at 70°C for 5 minutes.

2.5.14. Ligation Reactions

T4 DNA ligase was used to ligate DNA fragments with compatible cohesive or blunt-ended termini. The fragment of insert and vector were mixed at a ratio of 3:1 (insert:vector). 0.2 volumes of 5x ligase buffer (supplied with the enzyme) was added and

1 unit of DNA ligase added. This was incubated overnight at 4°C. 1 to 10 µl of the ligation reaction could be transformed into competent *E. coli*.

2.5.15. 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 technique takes advantage of Exonuclease III's requirement for double stranded DNA; the Exo III nuclease will digest 3' termini from blunt and 5' protruding termini, but not 3' single strand overhangs.

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. The enzymes were removed using the Promega Wizard Cleanup system according to the manufacturers instructions. The Exo III reaction was set up as follows for each time point:

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

The reaction was started by adding Exo III, and incubation at the optimal temperature (Table 2.4) to yield the desired deletions. 25 µl of the reaction mixture was removed at the appropriate time and added to a tube containing 40 µl 5x Mung Bean Buffer and 135 µl dH₂O, and then placed on ice. When all aliquots were removed, tubes were heated to 68°C for 15 minutes and placed on ice. 40 units Mung Bean Nuclease (diluted with 1x Mung Bean Dilution Buffer) were added to each tube, and incubated at 30°C for 30 minutes. The now blunt ended deletions were self ligated, and 7 µl transformed into *E. coli*. The 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 2.4. Progress of Exo III digestion at different incubation temperatures.

2.6. DNA Hybridisation Procedures.

2.6.1. Radiolabelling of DNA Fragments.

DNA fragments were labelled with a [α - ^{32}P] dCTP by the random primer labelling method using an Amersham Multiprimer kit. 30-50ng of the DNA to be labelled, in a total volume of 28 μl , were boiled for 5 minutes and then held on ice for 2 minutes. 10 μl of labelling buffer, 5 μl of random hexanucleotide primers, 5 μl ^{32}P -dCTP (equivalent to 50 μCi) were added, followed by 2 μl of Klenow enzyme. The labelling reaction was now left to proceed at room temperature overnight. The labelled DNA was boiled for 5 minutes immediately before use.

2.6.2. Southern Blotting.

DNA was transferred to Hybond-N (Amersham) nylon membranes, according to the manufacturer's instructions. The agarose gel containing the DNA samples was photographed with a ruler down its side. The gel was then covered with denaturation buffer (1.5M NaCl, 0.5M NaOH) and left with occasional shaking for 30 minutes. This was followed with two rinses in distilled water, then the gel was soaked in neutralisation buffer (1.5M NaCl, 0.5M Tris.HCl pH7.2, 0.001M EDTA) for a further 30 minutes. After rinsing the gel twice with distilled water, the blot could be set up. A reservoir of 10x SSC was set up, a platform was placed over this and a long piece of Whatman 3MM paper (presoaked in 10x SSC) put on this platform with its ends dipping into the reservoir. The gel was placed, wells uppermost, on the 3MM paper, and a piece of Hybond-N nylon membrane cut to the same size as the gel was placed on top of it. Any air bubbles were carefully removed before 3 sheets of Whatman 3MM paper, cut to gel size and presoaked in 10x SSC, were placed on top. Finally 2 layers of disposable nappies (also cut to gel size) were added and the stack covered with a glass plate and a 1kg weight placed on

top. The blot was left for at least 16 hours to allow the DNA to transfer. After which the apparatus was dismantled, the positions of the wells marked on the nylon and the DNA fixed onto the membrane according to the manufacturer's instructions. The filter was allowed to air dry for 1 hour before wrapping it in clingfilm and exposing it to UV light for two minutes.

2.6.3. Hybridisation of Radio-Labelled Probes to Southern Blots.

Hybridisation reactions were carried out using Techne Hybridisation tubes in a Techne Hybridiser HB-1 oven. The nylon filter was put inside the hybridisation tube and 200 μ l of pre-hybridisation solution (5x SSC, 5x Denhardt's solution [50x Denhardt's solution is 1% ficoll, 1% polyvinylpyrrolidone, 1% BSA fraction V], 0.1% SDS and 100 μ g/ml of denatured salmon sperm DNA) added per cm² of filter. Any air bubbles were carefully removed and the tube incubated at 65°C for 2 hours, whilst being rotated in the oven. The labelled probe was denatured, by boiling for 5 minutes, and added to the tube contents. The tube was now put back in the oven and incubated at 65°C for at least 12 hours. After incubation the pre-hybridisation solution (containing the probe) could be poured off or transferred to a glass bottle, if the probe was needed again.

2.6.4. Washing of Probed Southern Blots.

The nylon filter was kept within the hybridisation tubes whilst being washed and was never allowed to dry out. All washes were carried out at 65°C, the filter was washed for 15 minutes in 2x SSC, this was followed by a 30 minute wash in 2x SSC, 0.1% SDS. A final wash in 0.1x SSC was carried out for 10 minutes. After each washing solution was removed, the filter was checked with a Geiger counter and the washing continued until sufficient radio-labelled probe was removed. Finally the filters were wrapped in clingfilm.

2.6.5. Detection of Hybridising Probes.

Radioactive bands could be detected on the filter by exposing Fuji RX-100 X-ray film to the paper. The film sheets were pre-flashed once to sensitise the film, and the exposure carried out at -80°C, for varying amounts of time. Exposed films were developed with Ilford phenisol developer for up to 4 minutes and fixed with Kodak Unifix fixer for 2 minutes. The position of the wells on the filter were marked on to the film and the size of any hybridising fragments could be calculated using the original gel photograph.

2.7. DNA Sequencing.

2.7.1. DNA sequencing.

Double stranded DNA was prepared using a modified alkaline lysis preparation (see section 2.6.5.).

DNA sequencing was always carried out with an Applied Biosystems 373A DNA Sequencer using double stranded DNA templates. Usually fluorescently labelled universal M13 primers from the Applied Biosystems PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit were used, according to the manufacturer's instructions. Occasionally custom-synthesised primers (synthesised on an Applied Biosystems 381A DNA Synthesiser) were used in conjunction with the Applied Biosystems Taq DyeDeoxy™ Terminator Cycle Sequencing Kit.

2.7.2. DNA Sequence Analysis.

The DNA sequence generated were initially analysed using the Macintosh computer programmes DNA Strider™1.2 (Marek, 1988) and Sequencher. Primary DNA sequence analysis involved aligning DNA fragments, analysing restriction sites, creating graphical maps of the sequenced fragments, and translating all six hypothetical reading frames.

Additionally the sequence was computed against nucleic acid and protein databases to identify homologous regions. Sections of the DNA were manipulated using the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software Package mounted on the SEQNET VAX 3600 at SERC, Daresbury, U.K., and then compared against the GenBank and EMBL databases using the FASTA programme (Pearson and Lipman, 1988). Alternatively open reading frames (ORFs) in the DNA sequence were identified using DNA Strider, translated into their protein sequence and compared against the Owl database using the SEQNET programme SWEEP.

2.8. Transformation.

The transformation procedure used was a modified version of that described by D. Hanahan (Hanahan, 1983).

2.8.1. Preparation of Competent *E. coli* -- Calcium Chloride Competent Cells.

A single colony of *E. coli* was grown overnight in 5ml Luria broth at 37°C with agitation, 1ml of the overnight was added to 50ml Luria broth and grown for 2.5-3 hours at 37°C. Cells were transferred to 50ml Oakridge tubes and centrifuged at 7000 r.p.m. for 5 minutes in a Beckman J2-HS centrifuge using a JA-20 rotor. The pellet was resuspended in 25ml, ice cold 0.1M CaCl₂ and left on ice for 30 minutes. The cells were spun down as above and resuspended in 5ml ice cold CaCl₂. 3ml of 80% glycerol was added and 400µl aliquots of competent cells were distributed into eppendorfs and stored at -80°C.

2.8.2. Transformation of *E. coli*- Heat Shock.

Tubes of competent *E. coli* cells were thawed on ice and approximately 200ng of the DNA to be transformed was added. The tubes were mixed, incubated on ice for 30 minutes and then heat shocked at 47°C for 2 minutes. 1ml of Luria broth was added, the cells incubated at 37°C for 1 hour then aliquots plated onto selective media. Transformants were obtained after incubation overnight at 37°C.

2.8.3. Transformation of *S. cerevisiae* by Electroporation.

Procedure was based on a method from B. Stevenson (personal communication).

10ml YPDA were inoculated with a colony of the yeast strain to be transformed and incubated overnight with agitation at 30°C. 100µl was used to inoculate 100ml YPDA and incubated as above until a cell density of approximately 1×10^7 cells/ml was reached. The culture was centrifuged at 3000 r.p.m. for 5 minutes and the pellet resuspended in 20ml ice cold dH₂O. The cells were washed twice in ice cold 1M sorbitol by resuspending the pellet and spinning the cells down as above, before finally resuspending in 0.3-0.5ml of 1M sorbitol and storing on ice until transformation. Up to 5µl of DNA solution was premixed with 1µg of carrier DNA (single stranded Salmon sperm DNA). To this 100µl of cells were added, mixed and transferred into a cold gene pulser cuvette (Biorad 0.2cm electrode gap). The cells were pulsed at 1.5V (7.5kV/cm), 25µF, 400Ω for about 4.8ms (Biorad gene pulser.) 300µl 1M sorbitol was added to the transformation mixture and 100µl aliquots were plated out onto selective media. The plates were incubated at 30°C for at least 4 days.

2.9. Protein Biochemistry Methods.

2.9.1. Lämmlli SDS Polyacrylamide Gel Electrophoresis (Lämmlli, 1970).

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

Running gels were made using 10% acrylamide (see Table 2.5.). Acrylamide/bis, dH₂O, 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 pouring the running gel, it was overlaid with isopropanol and allowed to polymerize at room temperature for about 30 to 45 minutes. Isopropanol and unpolymerized acrylamide were poured off, the gel surface was washed with dH₂O and then overlaid with the 4% stacking gel. The comb was inserted and the gel allowed to polymerize for another 30 minutes. The comb was removed, the wells washed with dH₂O and the apparatus assembled for electrophoresis, filling both top and bottom reservoir with 1x PAGE buffer. Before loading samples were mixed with an equal volume of 2x sample buffer then boiled for 5 minutes and spun down before loading with Bio-Rad Prot/Elec tips. Gels were run at 30mA/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 | 10% | 4.0% |
| Acrylamide/bis stock | 8.325ml | 1.3ml |
| dH ₂ O | 10.425ml | 6.1ml |
| 1.5M Tris.HCl pH8.8 | 6.25ml | |
| 0.5M Tris.HCl pH6.8 | | 2.5ml |
| 10% SDS | 250µl | 100µl |
| 10% NH ₄ persulfate | 250µl | 50µl |
| TEMED | 25µl | 10µl |

Table 2.5. Formulation for SDS separating and stacking gels.

The acrylamide used in the gels was diluted from a 30% acrylamide stock which was made by the addition of 29g acrylamide and 1g N,N'-methylene bisacrylamide to 60ml distilled water. The solution was heated to 37°C to dissolve the chemicals and the volume then adjusted to 100ml with distilled water.

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

Cells were grown to an OD₆₀₀ 1.0 in YMM supplemented with appropriate amino acids and the equivalent of one absorbance unit was harvested and resuspended in 0.5ml yeast minimal medium, 50mM KPO₄ pH5.7, 2mg/ml BSA, and incubated at 30°C for 10 minutes. Tran[³⁵S]-label was added to a concentration of 100μCi/tube. The tube was incubated for 10 minutes at 30°C to label the proteins, then chased by adding 50μl methionine/cysteine (5mg/ml each) and incubated for another 30 minutes. The pulse-chase label reaction was stopped by the addition of 5μl 1M NaN₃. Cells were harvested and the supernatant transferred to a new tube containing 10μl 100x proteinase inhibitor cocktail (0.5mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin A). This tube represents the external fraction. 150μl freshly prepared sphaeroplast mix (1.4M sorbitol, 50mM Tris.HCl pH7.4, 2mM MgCl₂, 10mM NaN₃, 3μl/ml β-mercaptoethanol, 167U Lyticase/ml) was added to the cell pellet (internal fraction) and incubated at 30°C for 30 to 45 minutes. 100μl 10x IP buffer (0.9M Tris.HCl pH8.0, 1% SDS, 1% Triton X-100, 20mM EDTA) and 150μl dH₂O was added to the external fraction and heated to 100°C for 5 minutes. The internal fraction was supplemented with 10μl 100x proteinase inhibitor cocktail, 100μl 10x IP buffer and 519μl dH₂O and also incubated at 100°C for 5 minutes. After cooling to room temperature, each sample was pre-adsorped by adding 50μl IgSorb, 221μl dH₂O and incubating them on ice for 15 minutes. The tubes were centrifuged for 5 minutes at 12,000g and 950μl supernatant was transferred to new tubes containing 10μl anti-CPY antiserum diluted 1:10 in 1x immunobuffer (10mM Tris.HCl pH8.0, 0.1% SDS, 0.1% Triton X-100, 2mM EDTA). After 1 hour incubation on ice with occasional mixing, 50μl IgSorb was added to each tube, which were 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 1ml immunobuffer. Washed pellets were resuspended in 40μl 2x SDS loading buffer and then electrophoresed as described above (section 2.7.1.).

The gel was removed after electrophoresis and fixed in 25% Isopropanol, 10% acetic acid for at least 30 minutes, rinsed in water then soaked in 1M salicyclic acid for 15 minutes. The gel was dried at 60°C for 2 hours under vacuum then exposed to pre-flashed Fuji-XR film at -80°C overnight.

2.10. Light Microscopy.

2.10.1. Normarski Contrast.

Cells from a liquid culture could be observed under Normarski contrast by placing cells 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 Nikon F801s camera onto T_{max} film (400 ASA).

2.10.2. Indirect Immunofluorescence Microscopy.

Yeast cells were prepared for indirect immunofluorescence microscopy using a modified protocol to the one described by C.J. Roberts (Roberts et al., 1991).

Strains to be examined were streaked out to near confluence on fresh YPDA 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 OD₆₀₀ of about 0.25 in 10ml of YPDA and agitated at 30°C for 3 hours. The cultures were pre-fixed by the addition of 1.2ml of 37% (v/v) formaldehyde and agitation at 30°C for 60 minutes. Cells were centrifuged for 20 seconds and the supernatant removed, pellets were resuspended in 2ml freshly prepared fixative (4% formaldehyde, 0.1M KPO₄ pH6.5, 10mM MgCl₂.) and shaken at room temperature overnight. Cells were harvested, resuspended in 1ml 200mM Tris.HCl pH8.0, 2mM EDTA, 1% (v/v) β-mercaptoethanol and were transferred to microcentrifuge tubes. After 10 minutes at 30°C, cells were sedimented by low speed centrifugation in a minifuge for 20 seconds and washed twice in freshly prepared sphaeroplast solution (1.2M sorbitol, 50mM KPO₄ pH7.3, 1mM MgCl₂), before resuspending in 1ml of sphaeroplast solution supplemented with 150μg/ml Zymolyase and 25μl/ml glucylase. After 30 minutes incubation at 30°C with gentle shaking, sphaeroplasts were pelleted at slow speed and washed twice with 1.2M sorbitol. Pellets were resuspended in 1ml 1.2M sorbitol and treated with SDS. SDS denaturing conditions depended strongly on the primary antibody used, and are shown in Table 2.6.

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

Table 2.6. Denaturing conditions for cells prepared for indirect immunofluorescence with specific primary antibodies.

After denaturation, cells were washed three times in 1ml 1.2M sorbitol, and were finally resuspended in 1ml 1.2M sorbitol. A multi well slide was polylysine coated by adding 20 μ l of 1mg/ml polylysine to each well, after 20 seconds the wells were washed six times with water and allowed to air dry. 40 μ l of cell suspension was added to each well and allowed to settle for 10 minutes at room temperature. The fluid was aspirated off and the slide washed three times with 20 μ l PBS-BSA (1x PBS, 5mg/ml BSA,). 20 μ l PBS-BSA was added to each well and the slide left in a humid chamber for 30 minutes. The PBS-BSA was aspirated off and 10 μ l of the primary antibody solution added. Primary antibodies were used at a 1:10 dilution in PBS-BSA. After 1 hour incubation in a humid chamber the wells were washed six times with PBS-BSA and the second antibody was added. 10 μ l 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) was added and left for another hour. After six washes a 1:200 dilution of final antibody (FITC-goat anti-rabbit IgG) was added and the slide incubated for 1 hour as before. After nine washes, a drop of mounting medium (Citifluor containing 0.1 μ g/ml DAPI) was added and slide was covered with a 24 x 60 mm coverslip. After 5 minutes the two edges were sealed with nail varnish to fix the coverslip into position.

Slides were examined under an Nikon Optiphot-2 fluorescence photomicroscope (Nikon, Japan) fitted with filters for DAPI and FITC fluorescence. Each specimen was photographed under Normarski contrast (2 seconds), DAPI (4 seconds) and FITC (8, and 15 seconds) fluorescence onto T_{max} 400 film.

CHAPTER 3

CHARACTERISATION OF THE PLASMID pMLY38

3.1. Introduction.

The *ssv7-1* mutant was isolated by its inability to grow on 1.5M salt and characterised by M. Latterich (Latterich, 1992; Latterich and Watson, 1991). Preliminary characterisation of the *ssv7-1* mutant indicated that it had a moderate sorting defect. About 20% of the vacuolar protein CPY is mislocalised to the cell surface and it has a class B vacuolar morphology.

Utilising the inability of MLY1517 (*ssv7-1*) to grow on YPDA medium supplemented with 1.5M NaCl a cloning strategy was developed. Three plasmids from a YCp50 based yeast genomic DNA library were isolated. All three conferred salt resistant growth to MLY1517. The three plasmids; pMLY36, pMLY37 and pMLY38 were further characterised. pMLY36 and pMLY37 were shown by restriction enzyme mapping to contain overlapping genomic DNA, these were further analysed and the sequence of the *SSV7* gene was elucidated.

pMLY38 was originally shown to have an unrelated restriction pattern to pMLY36 and pMLY37. Initial studies suggested that pMLY38 not only restored growth under osmotic stress but also enhanced growth of the *ssv7-1* mutant strain (Latterich, 1992). This observation suggested that pMLY38 was a low copy enhancer/suppressor.

This study aimed to analyse further pMLY38 and to elucidate its role in relation to the *SSV7* gene.

3.2. Results.

3.2.1. Growth Behaviour of MLY1517/pMLY38.

pMLY38 was initially analysed to study its apparent enhancement of growth and complementation of the osmosensitive phenotype of the *ssv7-1* mutation. In order to verify this observation a comparison of growth curves was undertaken.

MLY1517 was transformed separately with pMLY36 and pMLY38 and the growth curves in YPDA + NaCl determined. As a comparison the growth curves of the wild type

clones was assembled using the computer programme, Sequencher (figure 3.4.). Restriction sites deduced from the nucleotide sequence agreed with the previously established restriction map (figure 3.4.).

3.2.3. Gene Disruption of *ORF1*.

ORF1 was disrupted by insertion of a *LEU2* cassette into the single *EcoRV* site (see figure 3.4.). pCH24 is a subclone of pMLY38 containing the 1kb *XbaI* 3' fragment in pUC19 (see figure 3.3.). This fragment contains the first 680 base pairs of the open reading frame. The gene disruption was carried out by cutting pL2¹ with *HpaI* and isolating the 2kb fragment containing the *LEU2* gene. pCH24 was cut with *EcoRV* which cleaves 591 base pairs into the open reading frame. The *LEU2* fragment was blunt end ligated into pCH24 and the resulting plasmid (figure 3.5) transformed into bacterial JA221 cells. These cells were grown on M9 media supplemented with thiamine, tryptophan and ampicillin but lacking leucine, allowed the selection of plasmids exhibiting the *LEU2* gene. The gene disruption was excised from the plasmid (pCH28) by digestion with *XbaI* and the resulting 3kb fragment purified from an agarose gel. This *XbaI* fragment was transformed into diploid wild type cells (6210.5) by electroporation. Colonies were selected for their growth on minimal media plates lacking leucine. In order to check that colonies had incorporated the *LEU2* gene disruption a Southern blot was performed. Total DNA was extracted from eight random colonies and the wild type parent diploid. These were digested with *XbaI* and separated on an agarose gel with the 1kb *XbaI* fragment from pCH24 as the control. The gel was blotted and probed using radiolabelled 1kb *XbaI* fragment. The results distinctly show that incorporation of the *LEU2* disrupted fragment has occurred. 6210.5 only shows one band at about 1kb, the same size as the undisrupted gene or the probe. The eight potentially disrupted strains give the expected two band pattern. One band highlighted at about 1kb, the undisrupted gene, and the other at 3kb indicating that the disruption has worked (figure 3.6.).

Two colonies were taken and patched onto YPDA agar for two days then replica plated onto sporulation agar and left for a week at room temperature after which 18 tetrads were dissected (figure 3.7.). The *ade*, *lys*, and *leu* auxotrophs and salt sensitivity were checked. Results showed that *ORF1* was not an essential gene as all 72 spores grew. Good segregation was observed with each phenotype segregating 2:2 per tetrad.

¹. pL2 is a derivative of pUC12 containing a *LEU2* cassette from yeast.

strain (SEY6210) and the untransformed *ssv7-1* mutant (MLY1517) in YPDA and YPDA + salt were also determined (figure 3.1.). Cultures were grown overnight in 10ml YPDA or minimal media supplemented with sufficient amino acids to maintain the transforming plasmid. The cell densities were calculated and an equal quantity of cells transferred to 100ml YPDA or salt supplemented media as indicated. All cultures were grown at 30°C and 140r.p.m.. 200µl samples were taken at regular intervals and the total cell number determined using a Coulter Counter. The results show the expected sigmoidal growth curve of the wild type and mutant cells grown in nutrient rich YPDA media. The mutant divides at approximately half the rate of the wild type strain (Latterich, 1992) thus explaining the difference in growth rate between the two. No growth of MLY1517 in salt media was observed. Contrary to the expected results pMLY38 did not confer an advantageous growth effect on MLY1517. Results of experiments from four separate occasions showed a similar trend. SEY6210, MLY1517+pMLY36 and MLY1517+pMLY38 when grown in salt supplemented media all grow at the same rate.

3.2.2. Sequencing of pMLY38.

A detailed restriction analysis of pMLY38 was carried out and the genomic insert calculated at 10kb. A restriction map was produced by digesting pMLY38 with a selection of enzymes. This information was used to produce five subclones in pRS316, a *URA3* amp^R centromeric shuttle vector (figure 3.2.). These subclones were transformed into MLY1517 and colonies tested for complementation of the *ssv7-1* mutation, by the ability to restore the osmosensitive phenotype (figure 3.2.). None of the subclones tested supported growth of the mutant on salt supplemented YPDA plates suggesting that the subclones did not cover the effective coding region.

pMLY38 was cut separately with *XbaI* and *EcoRI*, the three smaller fragments from the *XbaI* digest and five from the *EcoRI* digest were purified from an agarose gel and subcloned into pUC19. Sequence was determined using an ABI automatic sequencer (see Materials and Methods). Data obtained from the initial sequencing was analysed using the FASTA programme at the SEQNET, Daresbury. This showed that pMLY38 contained the *SSV7* gene. Alignment of sequence data using the Sequencher and DNA Strider programmes indicated a further open reading frame downstream of *SSV7* (figure3.3.).

This potential open reading frame (ORF1) was subsequently completely sequenced using subcloning and ExonucleaseIII - mung bean nuclease generated nested deletions. The open reading frame was sequenced in both directions, stretches of nucleotide sequence that could not be subcloned were sequenced by custom synthesising 18mer sequencing oligonucleotides from unambiguous parts of the open reading frame and using the oligomers in standard sequencing reactions. The nucleotide sequence of the overlapping

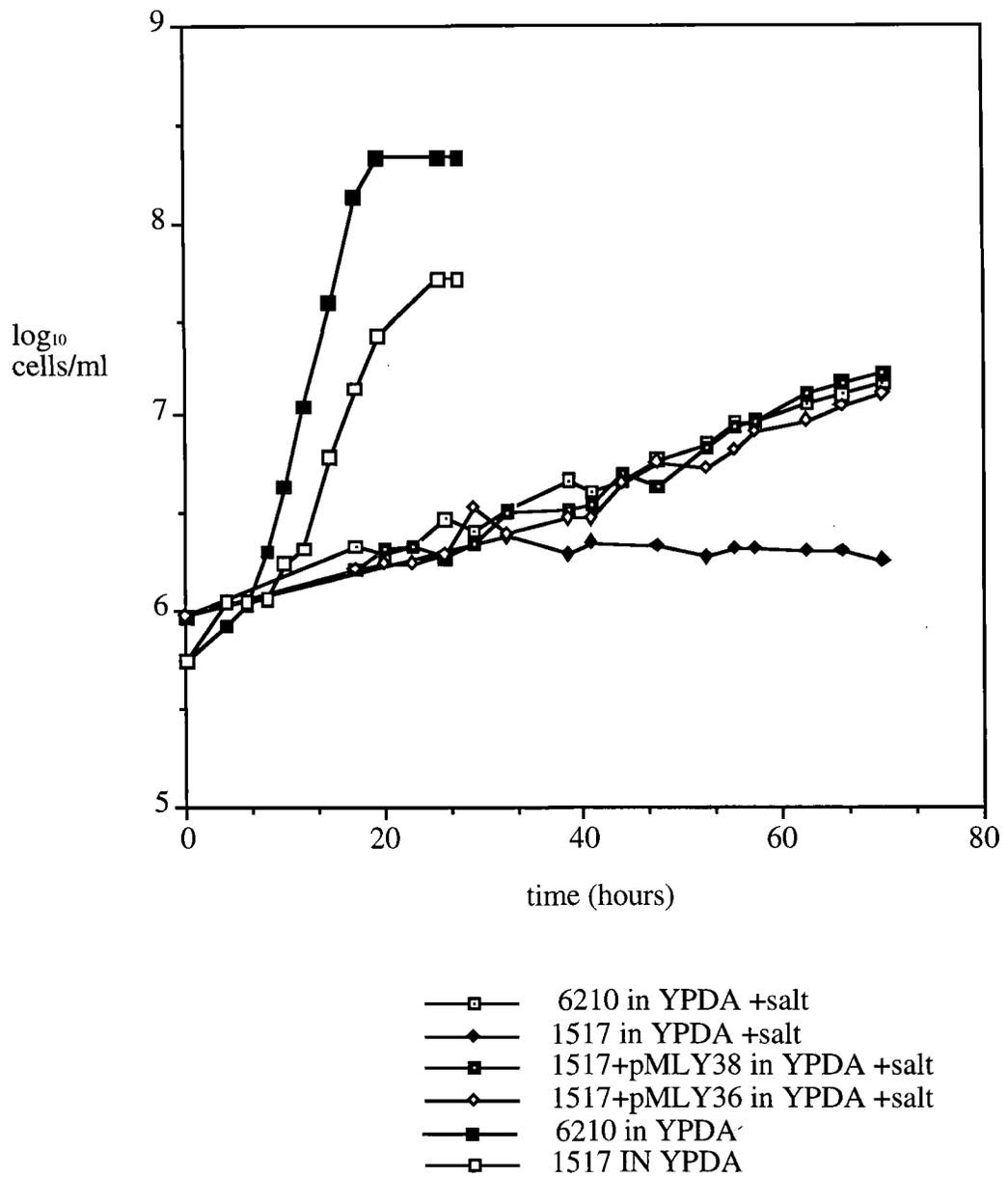


Figure 3.1. Growth curves of SEY6210 (wild type) and MLY1517 (*ssv7-1*).

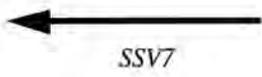
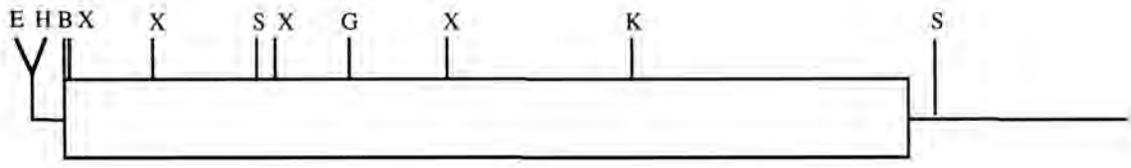
Graph shows growth curves of wild type and *ssv7-1* mutant strain under non-stressed conditions in YPDA media. It also shows the correlation between MLY1517 complemented with either pMLY36 or pMLY38 grown in 1.5M salt supplemented YPDA and the growth of the wild type and MLY1517 grown under similar conditions.

Figure 3.3. Initial sequencing and open reading frames "found" within pMLY38.

Three bands from the *Xba*I digest and 5 bands from the *Eco*RI digestion of pMLY38 were isolated and subcloned into pUC19. Each subclone was sequenced in both directions. Sequence data was aligned and analysed using FASTA searches and DNA Strider. This showed that pMLY38 contained *SSV7* and a further potential open reading frame (*ORF1*).

The arrows indicate the sections of the subclones sequenced. Above the arrows *Eco* or *Xba* indicate which enzyme was used to subclone the plasmid. The number indicates which fragment off the gel was isolated, the smaller the number the larger the fragment isolated, subcloned and sequenced. fp and rp refers to the forward primer and reverse primer respectively.

The figure shows *Xba*I and *Eco*RI fragments overlapping with each other and with *SSV7*. Analysis of the open reading frames was performed using the DNA Strider programme, this analyses all six frames numbered 3 to -3 along the side of the diagram. Short vertical bars in the open reading frame represent possible start codons (ATG), and long vertical bars the stop codons (TAA, TGA and TAG). The nucleotide scale is given by the numbers across the top of the box. Two open reading frames can be identified within the DNA initially sequenced, *SSV7* which is shown in the reverse direction in phase -3, and an open reading frame (*ORF1*) in phase 3 in the forward direction. The remainder of the *SSV7* sequence had been previously determined (Latterich, 1992).



Complementation
of ssv7-1

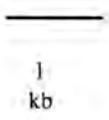
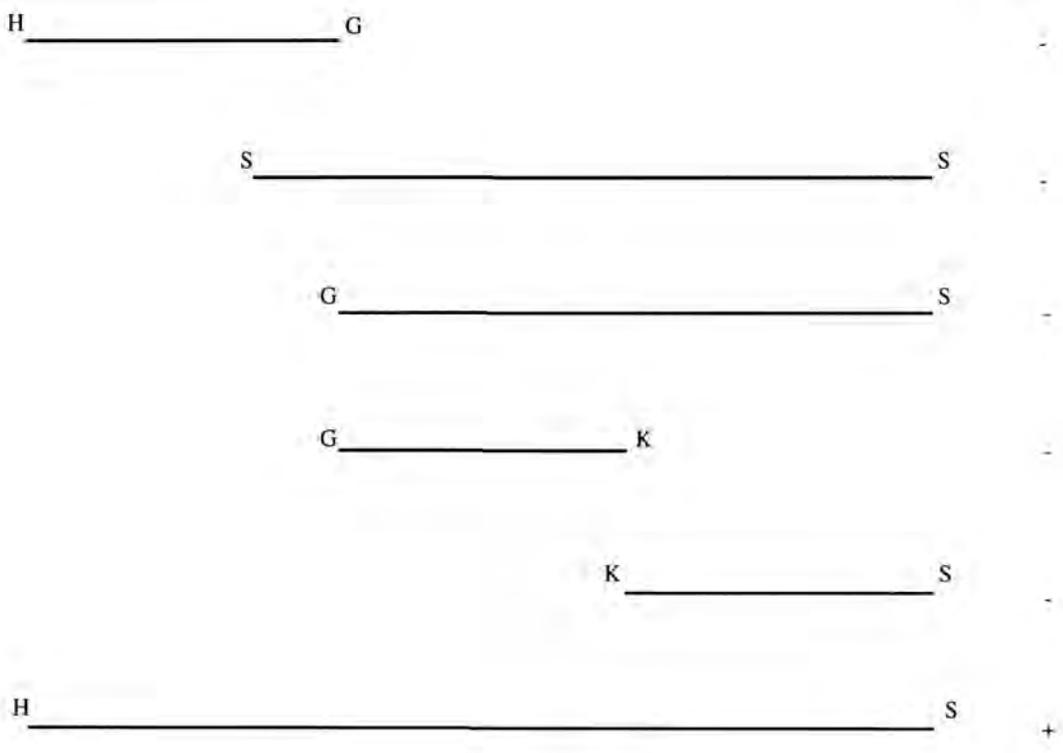


Figure 3.2. Preliminary restriction map and subcloning strategy to find the smallest possible complementing subclone of pMLY38.

pMLY38 was restriction digested in single and double digests and a restriction map constructed. Fragments of the genomic clone were subcloned into the yeast centromeric vector pRS316. Constructs were transformed into MLY1517, and URA3 transformants were tested for their osmosensitive phenotype. Subclones complemented the *ssv7-1* mutation if they were able to sustain growth on YPDA+1.5M NaCl. Only the *HindIII/SalI* subclone was able to complement the *ssv7-1* mutation.

The position and orientation of the *SSV7* gene found later to be present on the plasmid pMLY38 is represented by a bold arrow below the open box.

The restriction map at the top of the diagram shows the positions of the enzymes: E, *EcoR*I; H, *HindIII*; B, *Bam*HI; X, *Xba*I; S, *Sal*I; G,*Bgl*II; K, *Kpn*I. The open box represents genomic DNA inserted in the yeast vector YCp50.

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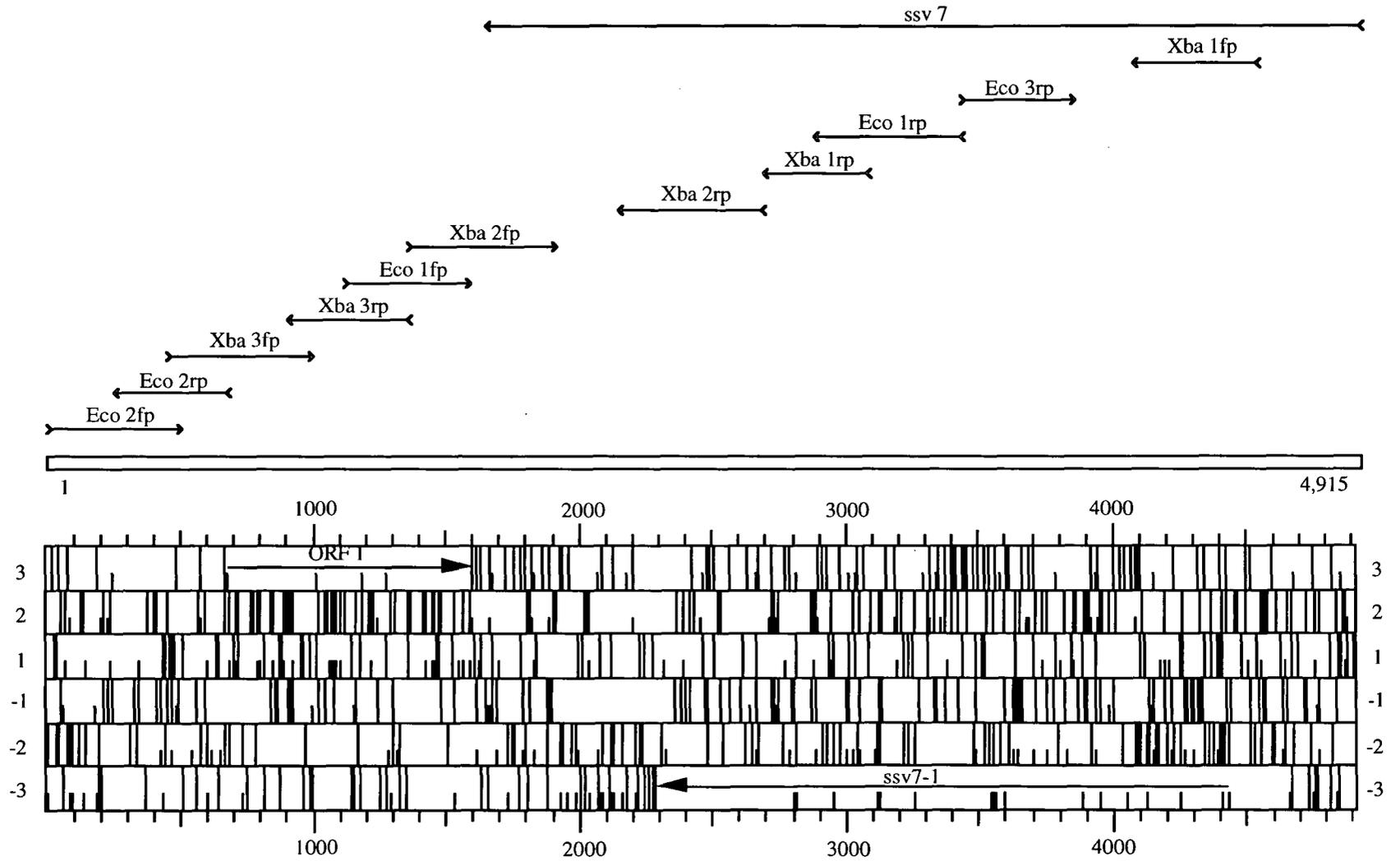


Figure 3.4. Sequencing strategy and restriction map of *ORF1*.

Subclones of the genomic clone containing *ORF1* were sequenced using standard forward and reverse sequencing primers. Some sequences were established using custom synthesized oligonucleotides as primers based on regions of known sequence. Data produced from these oligonucleotides are represented by black circles.

The dark box represents *ORF1* which has been expanded to show the restriction enzyme map. A gene disruption was subsequently carried out inserting a *LEU2* gene into the *EcoRV* site (figure 3.5.).

Open boxes represent flanking sequences. The narrow line represents the vector YCp50.

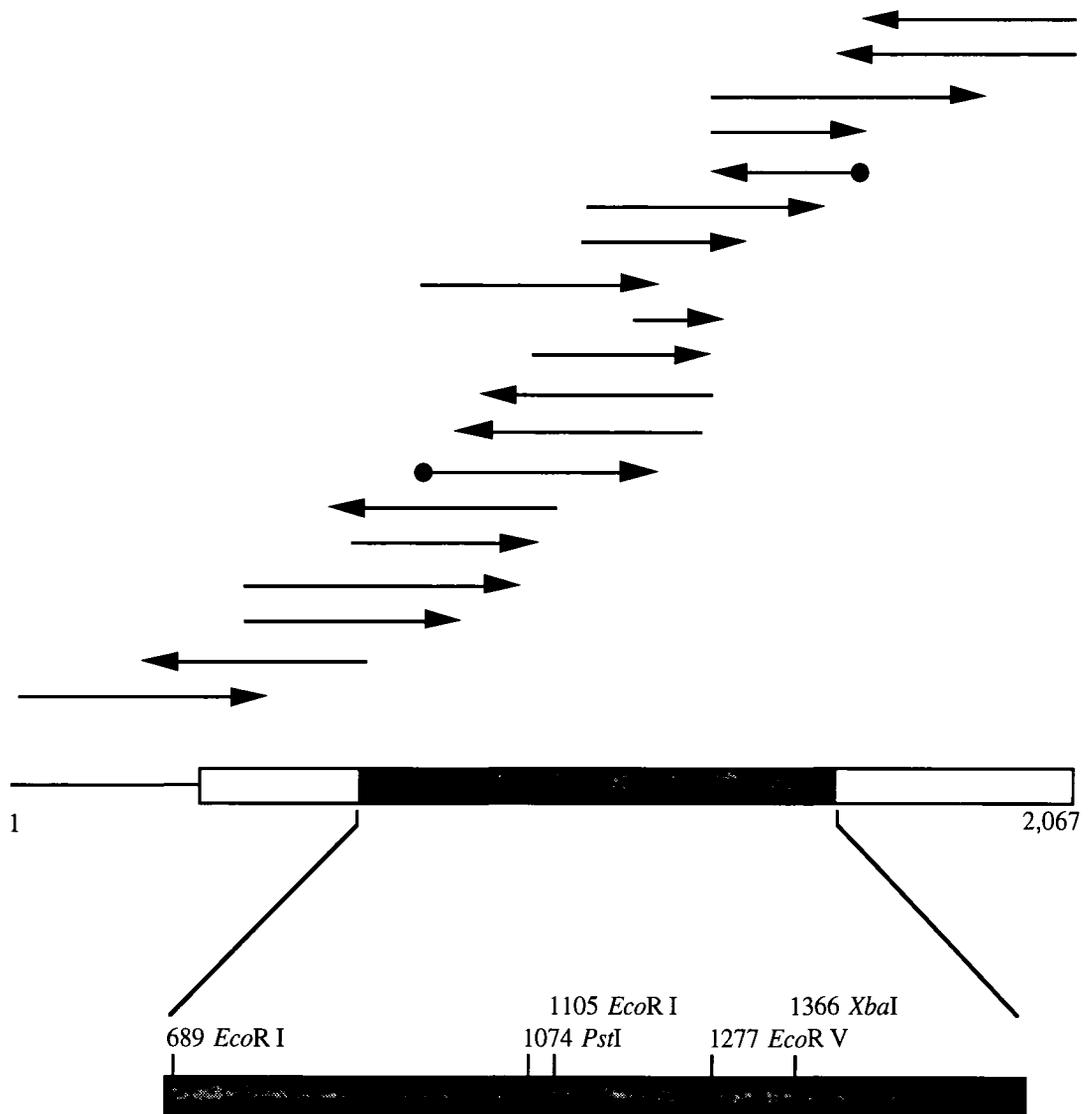
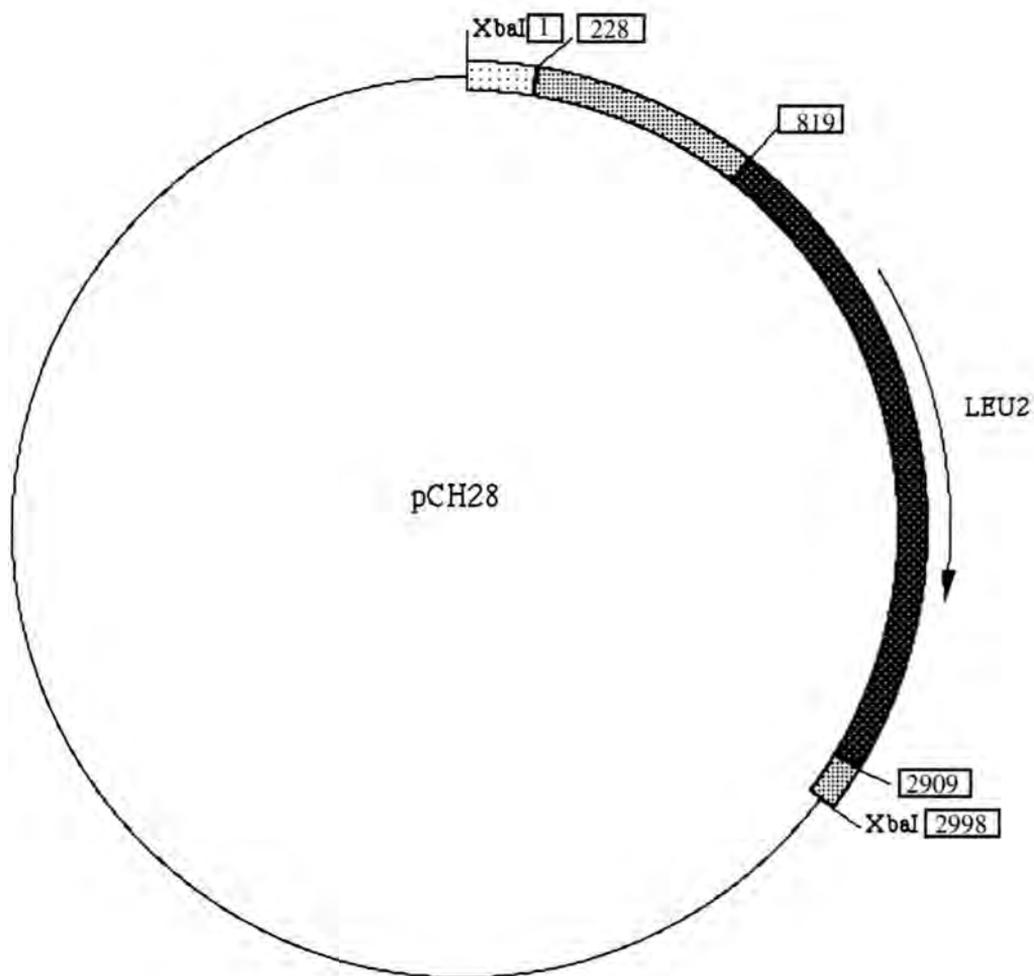


Figure 3.5. *ORF1* gene disruption.

ORF1 was disrupted by the insertion of a 2kb fragment containing the *LEU2* gene isolated from pL2 by digestion with *HpaI* and inserting into a unique *EcoRV* site in pCH24. pCH24 contains the 900bp *XbaI* fragment from pCH38.

The dark box represents the *LEU2* fragment. The dark shaded box shows the ORF within pCH24 and the light shaded box the remainder of pCH24 not within the ORF. The single line represents pUC19.



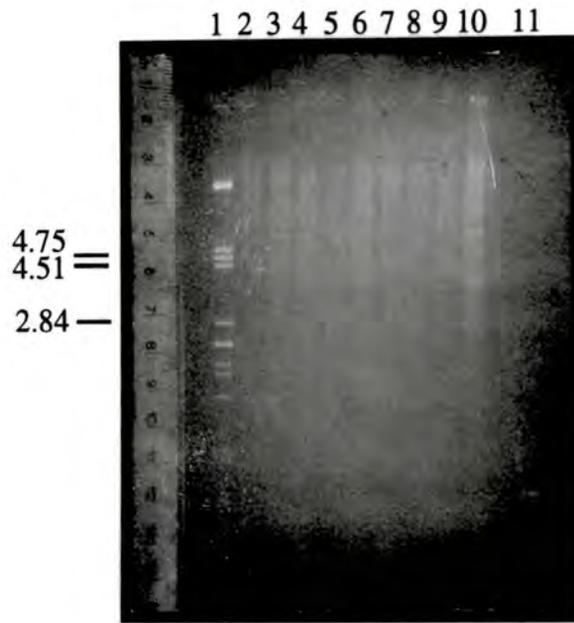


Figure 3.6.A. Agarose gel of *Xba*I digested total DNA.

Lane 1 shows the λ *Pst*I ladder. Lanes 2-9 contain total DNA extracted from diploids harbouring the gene disruption digested with *Xba*I. Lane 10 contains DNA from the wild type diploid digested with *Xba*I. Lane 11 contains the isolated *Xba*I fragment from pCH24 showing the size of the non-disrupted gene.

3.3. Discussion.

The plasmid pMLY38 initially thought to contain a low copy growth enhancer and suppressor of the mutant *ssv7* mutation, was sequenced and shown to contain a copy of the *SSV7* gene and a previously unidentified open reading frame (ORF1).

Initial studies looking at the growth of MLY1517 transformed with pMLY38 on solid media suggested that pMLY38 complemented MLY1517 and caused enhanced growth of the mutant cells on salt supplemented media (Latterich, 1992). This study used growth curve experiments accurately plotting the growth of cells in liquid media over a given time course, and comparisons were made with both wild types and mutants. This work indicated that no advantageous growth effect was conferred by the plasmid in YPDA supplemented with 1.5M salt. This is contrary to previous observations (Latterich, 1992).

A subclone analysis was undertaken to study which part of the 10kb insert complemented MLY1517. Six subclones were constructed. Only one, which contained the whole insert, complemented MLY1517. Later analysis showed a copy of the *SSV7* gene was present within pMLY38 and none of the five smaller subclones contained the whole gene, accounting for the failure in restoration of the salt resistant phenotype to MLY1517.

The growth experiments and subclone analysis suggests that the restoration of salt resistance to MLY1517 is due to the presence of a copy of the *SSV7* gene in pMLY38. Therefore the initial hypothesis that pMLY38 contained an enhancer or suppressor of the *SSV7* mutation is unfounded. However a previously unidentified open reading frame (ORF1) was discovered downstream of *SSV7* and subsequent analysis of this gene was undertaken.

ORF1 was sequenced in both directions and a restriction enzyme map established. This was used to produce a gene disruption of *ORF1* inserting a *LEU2* cassette into a single restriction enzyme site within *ORF1*. The disruption incorporated into the wild type diploid was verified by Southern blot and the resulting strain sporulated and the spores dissected. All spores grew indicating that *ORF1* is a non-essential gene.

ORF1 is found downstream of *SSV7* on the right arm of chromosome IV (Pearson and Lipman, 1988). Using the DNA Strider programme the open reading frame was translated into an amino acid sequence (figure 3.8.). A coding region of 933 base pairs, 310 amino acids, was identified with a predicted molecular mass of 35942 Da.

Initial analysis of the amino acids indicated a large proportion of acidic amino acids. Glutamic acid and aspartic acid constitute 14.8% and 12.2% of the total number of amino acids. An acid-basic map (figure 3.9.) shows a high concentration of acidic residues at the C-terminal end of the protein. Preceding this is a small basic stretch of amino acids.

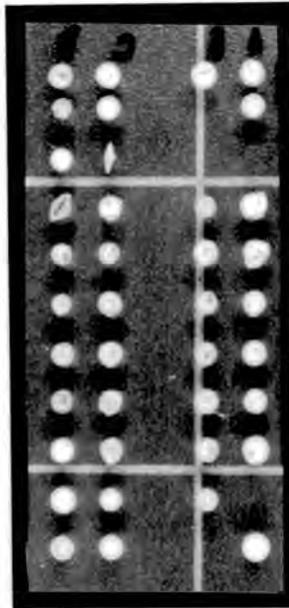


Figure 3.7. Spore dissection of the ORF1 gene disruption.

The diploid 6210.5 with the integrated ORF1 gene disruption was patched onto YPDA agar and grown for two days at 30°C before being replic plated onto sporulation agar and incubated at room temperature for seven days. Tetrads were dissected using a micromanipulator.

Disruptants

6210.5 Control

2 3 4 5 6 7 8 9 10 11

2990bp —

900bp —

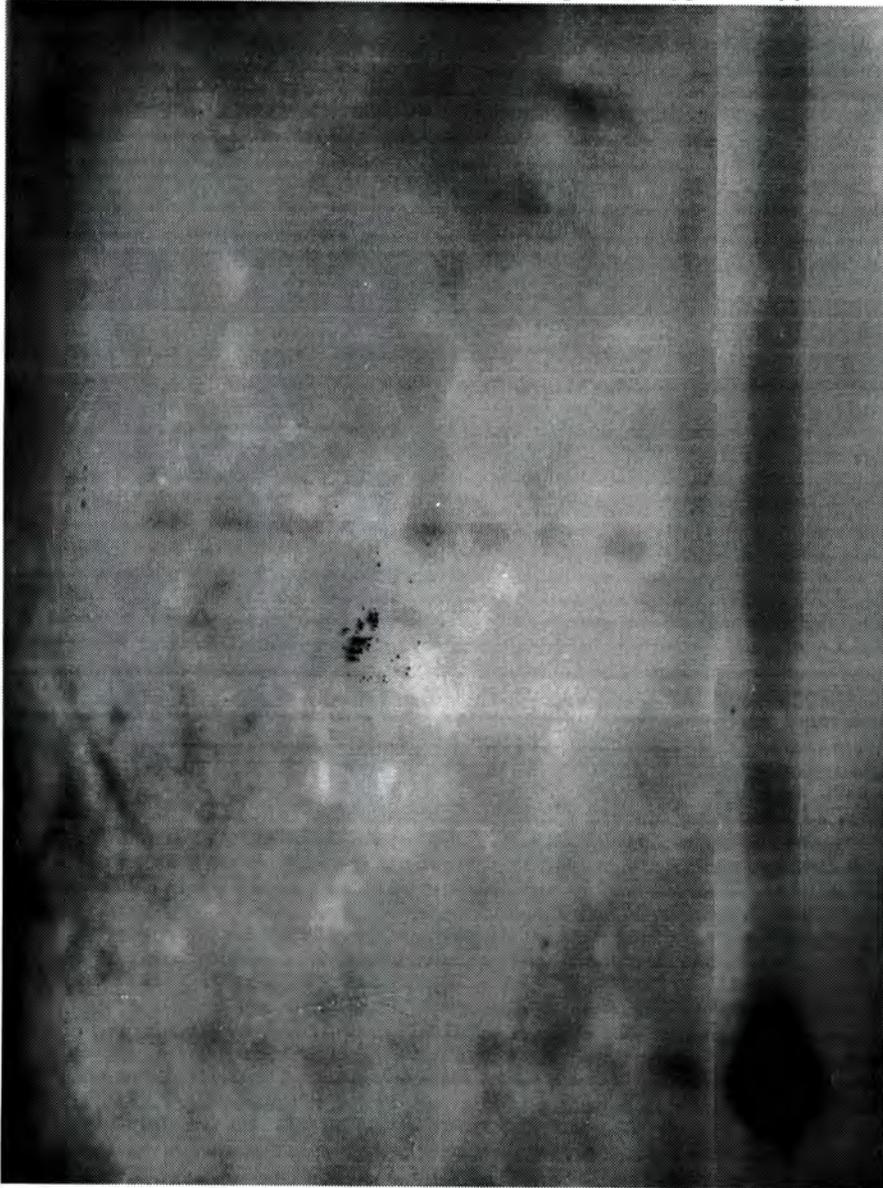


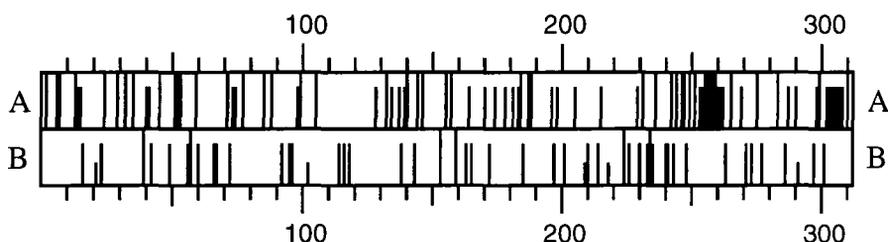
Figure 3.6.B. Southern blot of gene disruption.

Bands 2-9 show total *Xba*I digested DNA extracted different colonies of the disrupted diploid 6210.5. Lane 10 shows *Xba*I digested DNA from untransformed 6210.5 cells. Lane 11 contains a sample of the *Xba*I fragment used for the probe.

The Southern blot shows one band in lane 10 and two bands in lanes 2-9. The band in lane 10 corresponds to the non-disrupted gene as highlighted in control lane 11. Of the two bands highlighted in lanes 2-9 the smaller band corresponds to the undisrupted gene in lane 10. Comparison of the λ *Pst*I markers on the UV illuminated gel show that the second band in lanes 1-9 correspond to an increase in size of 2kb, which is consistent with the insertion of the LEU2 fragment into the *ORF1* gene.

Figure 3.9. Acid+Basic map of ORF1.

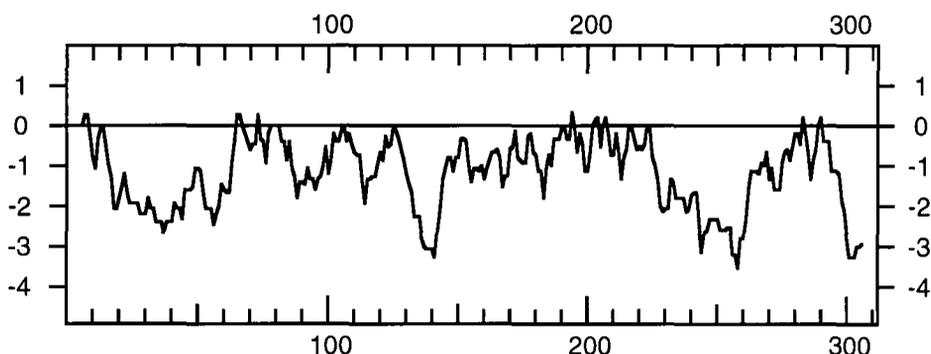
A refers to Acidic residues and B to Basic ones. The heights of the lines refer to the relative acidity/basicity of the residues.



Between amino acids 253-262 a highly acidic region consisting of five glutamic acid and five aspartic acid residues can be seen. Another region of interest is seven consecutive glutamic acid residues, at amino acids 302-308 at the end of the protein.

The hydropathy profile, according to the Kyte and Doolittle algorithm (Marek, 1988), of the putative protein is shown below in figure 3.10. This shows that the whole protein is hydrophilic.

Figure 3.10. The hydropathy profile of ORF1 using the Kyte and Doolittle analysis.



A search of proteins in the OWL database provided no significant homology to any proteins in the database. However, over a 134 amino acid stretch a 22.4% identity was found to a protein SCYKL202W1. This is a putative open reading frame from *Saccharomyces cerevisiae*, identified by the yeast genome project. The open reading frame is situated on the left arm of chromosome XI. ORF1 also has, on average, 24% identity over a 100 amino acid stretch to Nucleolar Transcription Factor 1 (upstream binding factor 1 - UBF1) from mouse, rat, human and *Xenopus Laevis*. The significance, if any, of these homologies is unknown.

Figure 3.8. Genomic nucleotide sequence of *ORF1* and the predicted amino acid sequence.

Figure shows *ORF1* and its predicted amino acid sequence. Of note is the high acidic amino acid content at the C-terminal of the protein. The underlined stretches of amino acids, 253-262 show a highly acidic stretch consisting of five glutamic acid and five aspartic residues. Between 302-308 lies a stretch of seven consecutive glutamic acid residues.

| | | |
|------|--|------|
| 1 | ggatcctctatataaatttggctgtacagtcgaaaattgtaacggagtcactagtgcat | 60 |
| 61 | tttgatacatatagcttattctagatgtgtgaactgggtattcatagcaattgtgaacc | 120 |
| 121 | atagtttatattggtactcctttccttcttaacagagtctcttttttctttcgcatttt | 180 |
| 181 | ttttggccttacatcaaatagcggtgattaatgataaaaagcacttagcagaagtcatgc | 240 |
| 241 | aacaaaaaaaaagaacacaacataagggtacagctagtgagctccattgcttcgacgtttta | 300 |
| 301 | ggataaa ATG GAA TTC TTT TAT GAA GAA CAA GTA GCT TGT ATC GAA | 346 |
| 1 | M E F F Y E E Q V A C I E | 13 |
| 347 | GAT GAT AAA ATA AGC AAC TCT CAT ACC AAG GAA ACT GGA TCA ACA | 391 |
| 14 | D D K I S N S H T K E T G S T | 28 |
| 392 | GAA AAC ACT GAA AAT AAC GAA CTT CAG AGC CGA GAT GAC AAA ACA | 436 |
| 29 | E N T E N N E L Q S R D D K T | 43 |
| 437 | AAT GAG GCC TTC CAA AAG TTA GAA GAG GAA GTC AAT AAA CGA TAT | 481 |
| 44 | N E A F Q K L E E E V N K R Y | 58 |
| 482 | GAA AAG ACT ACA AGT GCA TTC AAA AAG TTA GTA ATC GAG AAA GAT | 526 |
| 59 | E K T T S A F K K L V I E K D | 73 |
| 527 | GAC GGA ATT GAA ATT AAC TTG CCA ATT AGC AAT GAA ACT ACA GAG | 571 |
| 74 | D G I E I N L P I S N E T T E | 88 |
| 572 | ACT GCA CAA AAG TAT TTG AAG AAA CTA GAC GAG AAT ATT CAC AGC | 616 |
| 89 | T A Q K Y L K K L D E N I H S | 103 |
| 617 | GTG GAA AGT CTA GCC CAG TCA TAT TGG AGC AAA ATG AAA ACT AAG | 661 |
| 104 | V E S L A Q S Y W S K M K T K | 118 |
| 662 | AAT TTT TGG TCT GGC TTC AGT AGC TTC GAT AAT GCT GCA GAA AAT | 706 |
| 119 | N F W S G F S S F D N A A E N | 133 |
| 707 | GAC TCT AAT GAC AAG GAT GAG AAT TCG AAA GAA AAT GAA ATT GCT | 751 |
| 134 | D S N D K D E N S K E N E I A | 148 |
| 752 | GTG GGT GGA AAT AGA ACA GAA GCC GAA CTA AGG ACA TTA TCT AAA | 796 |
| 149 | V G G N R T E A E L R T L S K | 163 |
| 797 | GAT AAA TCG GTT TAT TTA GAC AAT AAA ATG GAT TTG CAA CTG GAC | 841 |
| 164 | D K S V Y L D N K M D L Q L D | 178 |
| 842 | CCG TTT GAC GTG GAT GAA AAA ACT GAG GAG ATA TGT TCT ATT TTA | 886 |
| 179 | P F D V D E K T E E I C S I L | 193 |
| 887 | CAG GGC GAC AAA GAT ATC TCC AAA TTA ATG AAC GAC ATC GTA CCC | 931 |
| 194 | Q G D K D I S K L M N D I V P | 208 |
| 932 | CAT AAA ATC AGC TAT AAA GAT TTC TGG CAC ATC TAT TTC TTA CAA | 976 |
| 209 | H K I S Y K D F W H I Y F L Q | 223 |
| 977 | AGA AAC AAA ATT CTA GAT AAA GAA AGT AAA AGG AAA GAA ATA TTG | 1021 |
| 224 | R N K I L D K E S K R K E I L | 238 |
| 1022 | TCC AAA AAG GAA AAG GAA ACG GAG GAA AAA GAA GTT GAG TGG GAT | 1066 |
| 239 | S K K E K E T E E K E V E W <u>D</u> | 253 |
| 1067 | GAC GAA GAA GAA GAA GAA GAT GAT GAC AAA GTA GAA GCA GTA GCT | 1111 |
| 254 | <u>D E E E E E D D D</u> K V E A V A | 268 |

| | | |
|------|---|------|
| 1112 | GAC AAC AAA TCT AAA GGG GAG ACG AAA GTT GCT GTG TCT CAA GAG | 1156 |
| 269 | D N K S K G E T K V A V S Q E | 283 |
| 1157 | GGA TTG AAA GAT GTT AGT GAC CAT GTT GGC CTC GCC AAC AAG GAT | 1201 |
| 284 | G L K D V S D H V G L A N K D | 298 |
| 1202 | GAG AGC AAG GAC GAC GAC GAC GAT GAT GAC TGG GAA TGA gtgatttt | 1248 |
| 299 | E S K <u>D D D D D D D</u> W E * | 311 |
| 1249 | tttcatataaaagatatatgtaatgctataaatatttacatacttttcatgacgtattat | 1308 |
| 1309 | ggtagaaaggaaaatcatcaatccaagagatgcacaggaggaagacaaaatctgggcgtg | 1368 |
| 1369 | atctcatctttacaccttctaaaaaaaaagttcgtttgtttaccggttaaaataaatcatttg | 1428 |
| 1429 | tatttaggggttttttatttgaccttagatggaaataatgatatgccatggtgaacgggaa | 1488 |
| 1489 | aaaaagtgtatagacaacgggttttcagttattttattcaaatgaatcaaacaccgtagacy | 1548 |
| 1549 | cggtgataaaaacaaaacgtatgcattagagttaattgcatcggtttgtttttgacaggt | 1608 |
| 1609 | ttatatttggtatcatcaaaatatagccatcccttctttaatccctttttcacataggct | 1668 |
| 1669 | gtataatgaccaccatacaaaagtaccaaagtggcatgctacaccatataattcactta | 1728 |
| 1729 | aaaggtggtattttgtccccttattggtagttcatcgtcattaacacctggaggaaaaacc | 1788 |
| 1789 | ccgtcaaaatcattggcccaaaatggagtcaagtccaacaaaaagggtatatgacgaag | 1848 |
| 1849 | tcattatTTTTGTttaaataaattatcaaatctctttaaTGGactatcagat | 1900 |

CHAPTER 4

ISOLATION AND CHARACTERISATION OF SSV16

4.1. Introduction.

The *ssv* mutants were isolated and characterised by Martin Latterich (Latterich, 1992; Latterich and Watson, 1991). 17 complementation groups were identified and phenotypic analysis undertaken. *ssv16-1* proved to have some interesting characteristics. MLY1156 (*ssv16*) complemented the other *ssv* mutants and the previously identified *vps* and *osm* mutants. It also fails to grow on other high osmotic strength compounds such as 2M Sorbitol. Unlike a number of previously identified *vps* mutants which failed to grow on non-fermentable carbon sources such as glycerol, MLY1156 does not display this Pet phenotype.

Electron microscopy revealed a class B vacuole. Secretion of proteins was determined using several methods. Colony blotting revealed that MLY1156 secretes CPY, PrA and PrB. Immunoprecipitation after pulse chase allowed quantification of protein secretion. MLY1156 secretes 80% of CPY, 10% PrA and only 1% PrB. CPY is secreted as the 68kDa p2CPY precursor, the mature 61kDa protein is not secreted. This suggests that mislocalisation occurs before vacuolar localisation.

Production of heterozygous diploids of MLY1156 harbouring the preproCPY-Invertase fusion plasmid which in the mutant haploid is mislocalised to the cell surface showed that the *ssv16-1* allele was not fully complemented by the wild type allele. *ssv16-1* still exhibited 20% secretion of the CPY-Invertase fusion protein in the heterozygous diploid. Therefore the *ssv16-1* phenotype was described as semi-dominant for secretion.

The interesting phenotype of *ssv16* in that it secretes a large percentage of CPY and its semi-dominance for secretion but recessive osmosensitivity made this mutant interesting to study. The ease with which MLY1156 is transformed made it an ideal candidate to clone by complementation from a yeast gene bank.

4.2. Results.

4.2.1. Indirect Immunofluorescence.

To check the classification of MLY1156 as having class B vacuole morphology an immunofluorescence technique was employed. As this method only involves fixation of the cells by formaldehyde followed by probing with antibodies, the processes involved are not as harsh as those involved in preparation for Electron Microscopy which may produce artefacts. Two vacuolar membrane markers Pho8p and the 60kDa subunit of the vacuolar ATPase were used, these are probed for by three consecutive antibodies, the last of which is conjugated to FITC which can be viewed under fluorescence microscopy. The results showed that *ssv16* indeed exhibited class B vacuoles, as a large number of small vesicles could be seen within the cell (figure 4.1.). This observation was consistent using both primary antibodies and was backed up by vital staining with CDCFDA (data not shown).

4.2.2. Immunoprecipitation.

The secretion of CPY was studied by immunoprecipitation. A diploid was constructed by backcrossing MLY1156-2B (α , *lys*) with the wild type SEY6211 (*a*, *ade*) and isolating the diploid on minimal media plates lacking lysine and adenine.

A comparison of secretion of CPY in the heterozygous diploid, the haploid mutant MLY1156-2B and the wild type was undertaken. The results show that the wild type does not secrete CPY. The *ssv16-1* mutant shows intracellular mature CPY at 61kDa but a large proportion of the proenzyme is secreted. The missorting of only the 69kDa form of CPY to the cell surface indicates that mislocalisation occurs before vacuolar localisation where the PrA mediated proteolytic processing of CPY takes place. Immunoprecipitation of CPY from the heterozygous diploid shows intracellular CPY, but a proportion of the p2CPY is secreted and found in the extracellular fraction. This confirms that *ssv16* displays a semi-dominant phenotype for CPY sorting (figure 4.2.).

Figure 4.1. Immunofluorescence of MLY1156-2B.

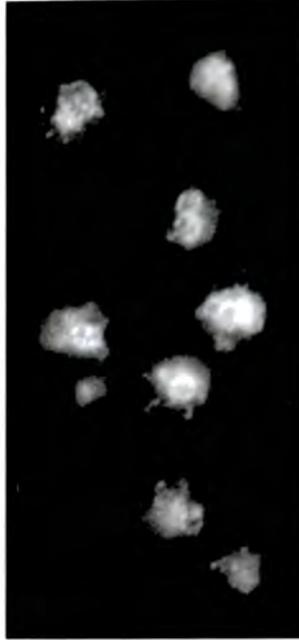
Pictures show immunofluorescence of MLY1156-2B showing the class B phenotype. The top three pictures are of the same set of cells viewed under different microscopic techniques. The first shows the cells under Normarski contrast optics indicating the cells outline. The second two photos are due to double staining of the cells, one with an antibody to *pho8* which is directed to the vacuole and the third picture is the fluorescence produced from addition of the vital stain DAPI, which lights up the nucleus. The bottom three pictures are the same except the antibodies used to highlight the vacuole are directed to the 60KDa subunit of the vacuolar ATPase.

Note the dispersed, vesicular pattern produced in by the *pho8* and 60KDa antibodies indicating a class B type vacuole pattern. DAPI staining is normal.

Normarski



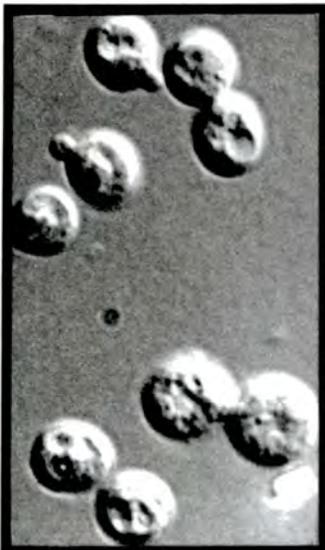
pho8



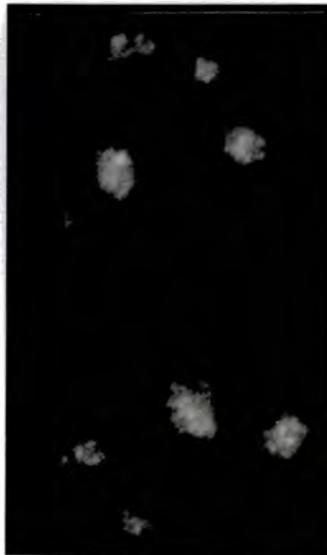
DAPI



Normarski



60KDa



DAPI



Figure 4.2. Immunoprecipitation of wild type, mutant and heterozygous diploids.

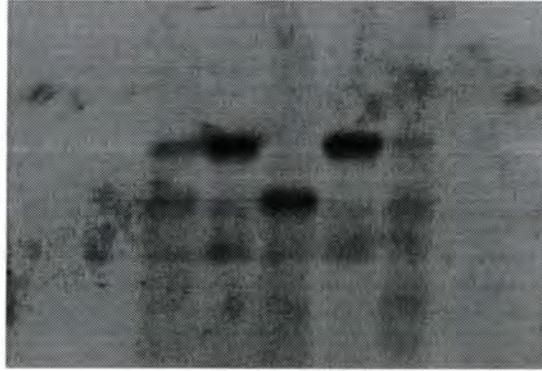
Immunoprecipitations were carried out on three strains. The wild type SEY6210 shown in lanes 5 and 6, The mutant *ssv16-1* strain MLY1156 in lanes 3 and 4, and the heterozygous diploid in lanes 1 and 2. Each strain shows immunoprecipitated CPY in the intracellular (I) and extracellular (E) fractions.

The two proteins highlighted by the CPY antibody are the mature CPY at 61kDa, which is located within the intracellular fraction, and the 69kDa proprotein which is the only form of CPY located in the extracellular fraction. The wild type shows no extracellular CPY whereas the *ssv16-1* mutant and the heterozygous diploid show CPY in both fractions.

Diploid MLY1156 SEY6210

1 2 3 4 5 6
I E I E I E

proCPY —
CPY —



4.2.3. Cloning of *SSV16*.

Although the *ssv16* mutation is semi-dominant for CPY localisation, it is recessive for osmosensitivity and therefore it is unable to grow on YPDA media supplemented with 1.5M NaCl. This was exploited to develop a cloning strategy to isolate the *ssv16* gene. MLY1156-2B (*ssv16*, *ura3-52*) cells were transformed with a YCp50 based genomic library (Rose et al., 1987) and URA3 transformants selected for on yeast minimal medium. At least 11,755 URA⁺ colonies were obtained. 10 colonies conferred salt resistant growth. The plasmids were reisolated from these salt resistant transformants and amplified in *E.coli* DH5 α . Three transformants failed to produce plasmids. Out of the seven others six of the plasmids appeared to be YCp50 and were discarded. The remaining plasmid, pCH30, was larger than YCp50 and on digestion with *Hind*III four bands were observed. This plasmid was reintroduced into MLY1156-2B and complementation of the salt sensitive phenotype checked. pCH30 on second transformation still appeared to confer salt resistance and further characterisation of this plasmid was undertaken.

A detailed restriction map was carried out digesting pCH30 with all available restriction enzymes (figure 4.3.). The insert was calculated to be 3kb and so complementation by subclones was not carried out. The plasmid was cut with *Xba*I and *Sal*I, there are two *Xba*I sites in the insert and *Sal*I cuts within YCp50. Two of the three fragments produced were cloned into pUC19 and sequenced using standard primers. Sequence data obtained was analysed using the FASTA programme at SERC Daresbury, U.K.. From the initial 560 base pairs analysed homology to a previously characterised gene was indicated. This initial search gave 95.9% identity to *SIS1*. Alignment of the remaining sequence data showed 100% identity to the *SIS1* gene. The restriction enzyme data already defined matched to that of the *SIS1* data. A restriction map and the open reading frame for the pCH30 insert was constructed (figure 4.4.) from the published data (GenBank/EMBL accession number: X58460).

4.2.4. Are *SIS1* and *SSV16* Identical.

In order to verify that pCH30 contained the cloned *SSV16* gene further experiments were undertaken. Following the initial work, subsequent transformation of MLY1156-2B with the plasmid pCH30 did not prove decisively whether or not complementation for salt-sensitivity occurred. pCH30 may not fully complement *ssv16-1* but act as a suppressor. Various experiments were undertaken in order to study the effect of pCH30 on the *ssv16-1* mutant.

Figure 4.3. Restriction map of pCH30.

The clone pCH30 was isolated from a YCp50 based gene library by its ability to complement the *ssv16-1* mutation. The insert, represented by a shaded box, was estimated at 3kb in length. This is within the 7.95kb YCp50 vector represented here by a single line. A restriction map was made and the positions on the insert calculated. Restriction enzymes are shown with the numbers being the base pair position relative to the start of the insert.

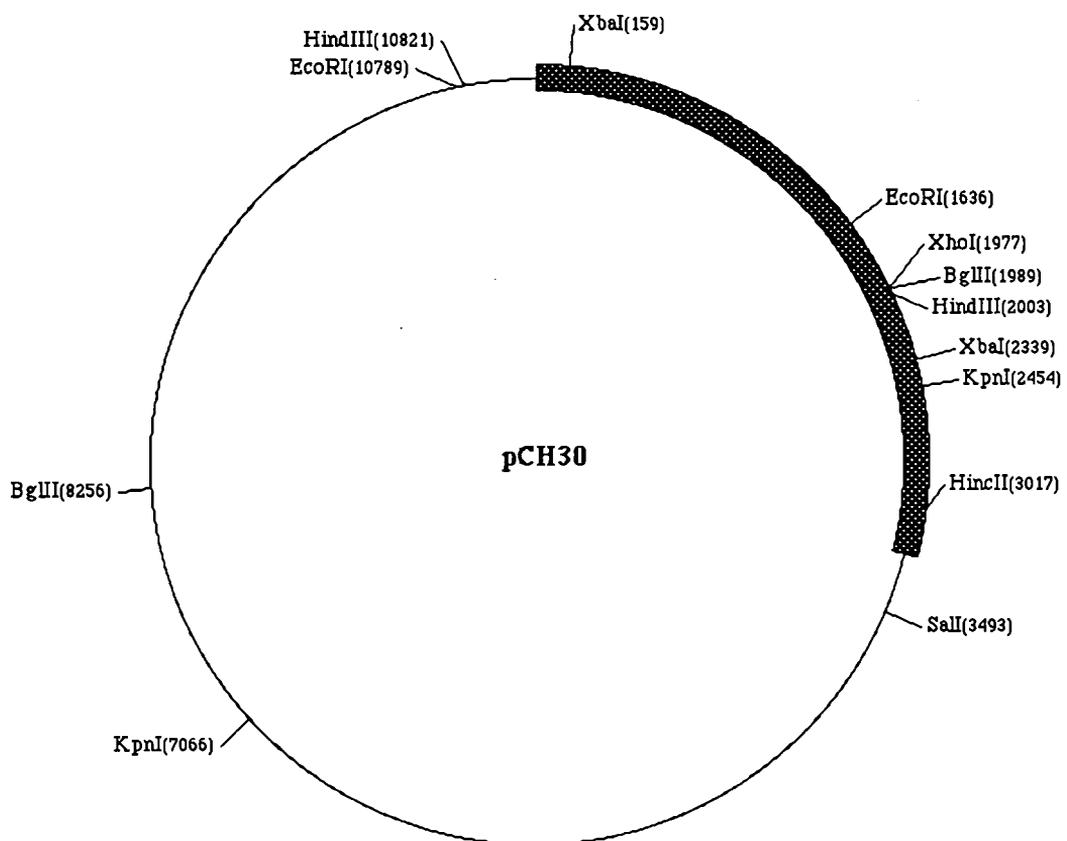


Figure 4.4. Sequencing strategy and open reading frames found within the insert of pCH30.

The shaded box represents the 3kb insert from pCH30 with the restriction enzymes cutting at the positions marked relative to the start of the insert. Above this is the map of the sequencing strategy with the position of the *SISI* gene marked for comparison.

Below is the analysis of the open reading frames in all six frames performed using the DNA Strider programme. The sequence data was taken from that published for *SISI* (GenBank/EMBL accession number X58460). One open reading frame can be identified in phase 3 which is about 1000bp in length.

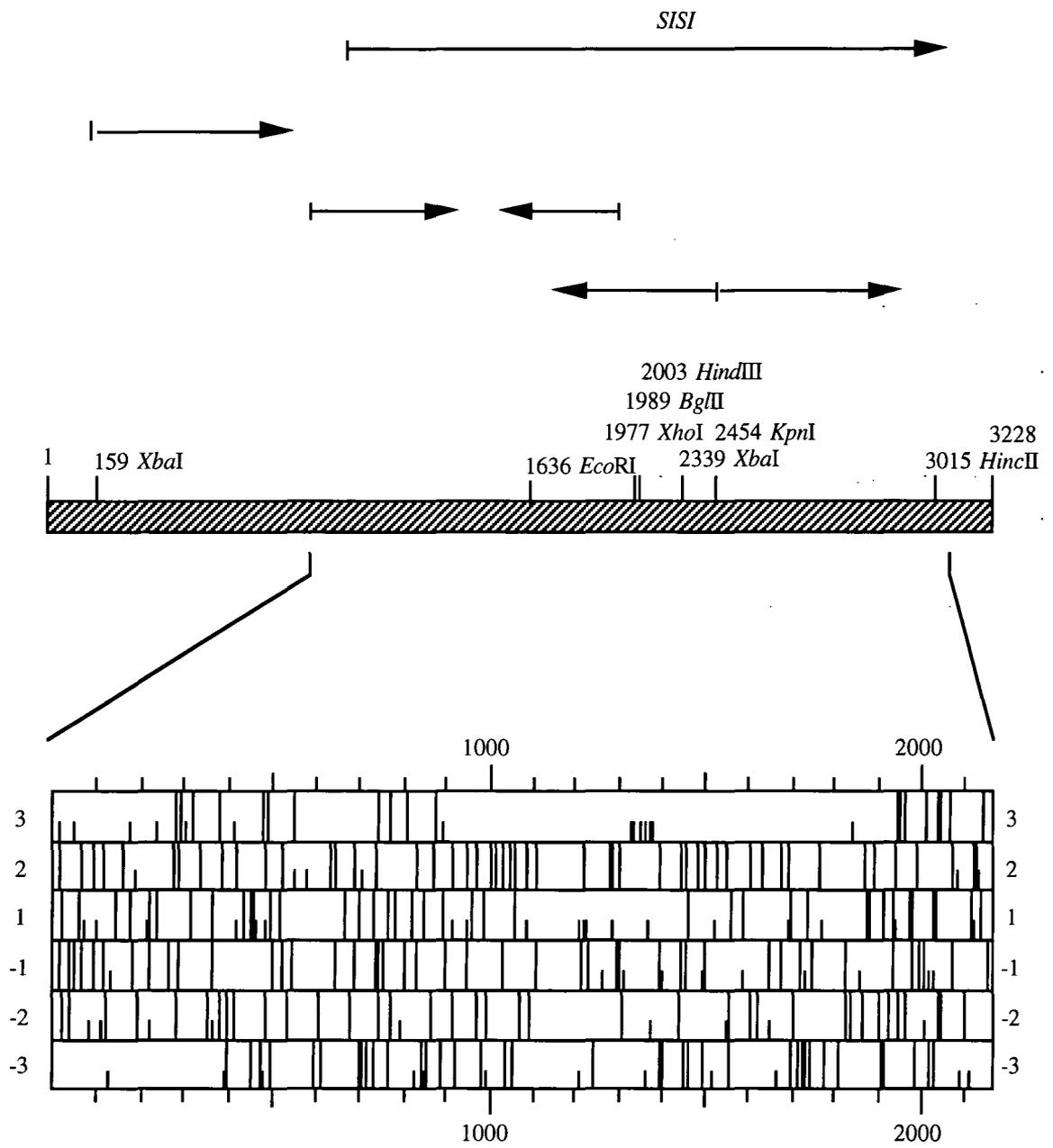
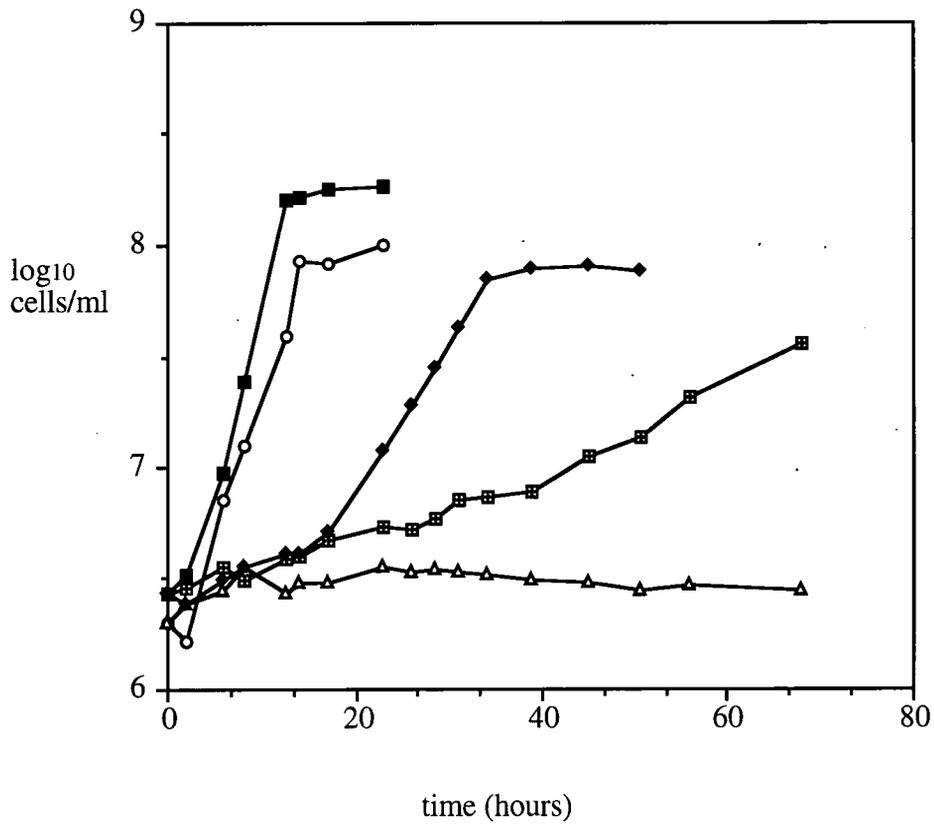


Figure 4.5. Growth curves of SEY6210 (wild type) and MLY1156 (*ssv16-1*).

The graph shows growth curves of the wild type and *ssv16-1* mutant strain under non-stressed conditions in YPDA media and stressed conditions in YPDA supplemented with 1.5M NaCl. It also shows the growth of the *ssv16-1* mutant in stressed conditions after transformation with the pCH30 plasmid.



A growth curve study followed the growth of MLY1156 transformed with pCH30 in YPDA+salt compared to the wild type and mutant MLY1156 in YPDA alone or YPDA+salt. 100ml of cultures were grown and aliquots counted at regular intervals in a Coulter Counter. The \log_{10} of the number of cells was plotted against time. The graph (figure 4.5.) shows normal growth for the mutant and wild type in YPDA. The wild type grows slowly in salt and the mutant shows no growth. The mutant strain transformed with the plasmid pCH30 grows more quickly in salt than the wild type strain. This experiment was repeated twice both sets of results showing a similar trend indicating that pCH30 does indeed suppress the *ssv16-1* mutation osmosensitive phenotype.

Immunoprecipitation using an antibody to CPY revealed that pMLY1156+pCH30 continues to secrete the immature p2CPY (figure 4.6.). This however is not surprising as the wild type *SSV16* gene itself fails to restore correct CPY localisation in the wild type/*ssv16-1* heterozygous diploid.

SIS1 is present on chromosome XIV (Luke et al., 1991). An investigation into the chromosomal location of *SSV16* was undertaken to help elucidate if *SSV16* was allelic to *SIS1*. A method of chromosome mapping via chromosome loss was employed (section 2.5.2.). This involved making diploids of the mutant MLY1156 with a set of yeast strains which subsequently lose a specific chromosome, thus revealing any recessive phenotypes on the monosomic chromosome. The 16 diploids were grown and after two subcultures in YPDA colonies were plated onto salt plates to test for salt sensitivity. The experiment proved inconclusive, on the second attempt *SSV16* appeared to map to chromosome II but two subsequent attempts failed to substantiate this observation.

4.3. Discussion

A mutation in *SSV16* causes a pleiotropic phenotype affecting a number of cellular processes such as vacuole morphogenesis, osmoregulation and protein targeting. The *ssv16-1* mutant has a vacuolar morphology characteristic of class B mutants as 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. An interesting feature to note is the semi-dominant phenotype exerted by the *ssv16-1* mutation in the diploid cells. This semi-dominance appears only to affect the protein targeting phenotype causing the secretion of a small proportion of the precursor CPY in *ssv16-1* heterozygotes. In order to study the mutation at the molecular level the gene was cloned and sequenced. The cloning was apparently successful and a 3kb fragment was subsequently analysed. This was shown to have a copy of a gene *SIS1* encoding a 352 amino acid protein (figure 4.7.).

Figure 4.6. Immunoprecipitation of *ssv16-1* +pCH30.

The figure shows the immunoprecipitation of four yeast strains, the wild type (SEY6210) lanes 1 and 2, MLY1156-2B (*ssv16-1*) transformed with pCH30 lanes 3 and 4, MLY1156-2B lanes 5 and 6 and the MLY1156/SEY6210 heterozygous diploid lanes 7 and 8. Each strain shows the CPY precipitated from the intracellular (I) fraction (lanes 1, 3, 5 and 7) and the extracellular (E) fraction (lanes 2, 4, 6 and 8). The two forms of CPY can be seen as described in figure 4.2.

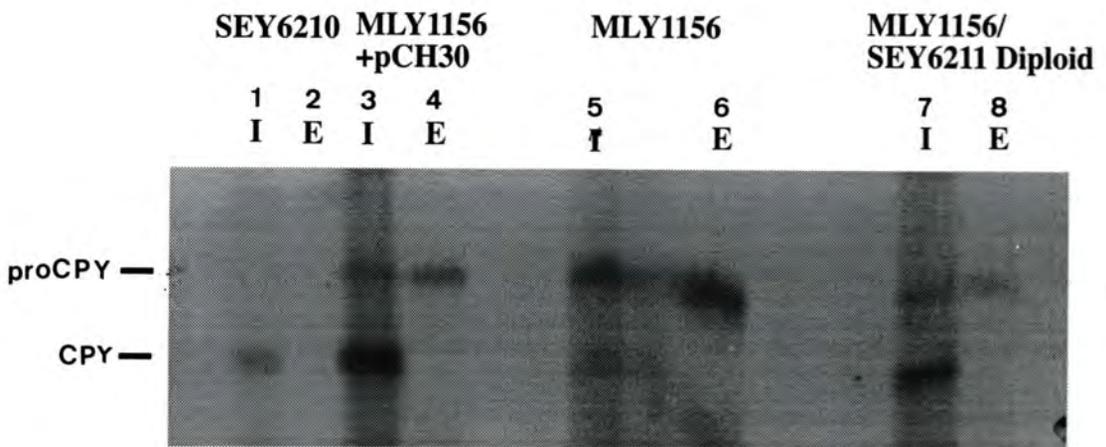


Figure 4.7. Genomic nucleotide sequence of *SIS1* and the predicted amino acid sequence.

Figure shows the sequence of *SIS1* as obtained from GenBank/EMBL, accession number X58460. The coding region starts at base pair 895 and is 1059 base pairs long, encoding a protein of 352 amino acids.

The four lines above the sequence data shows the extent of the sequence data obtained in this study. *SIS1* data from GenBank starts at base pair number 123 and finishes at 2159.

1 aactcgcctaggttaggatgaattacgacttcatttactggcggttatgtttgtaattt 60
61 ctaggaatggacggagagaacgcacatcccaaactttaatgggccatcttcgctagaggtc 120
121 accatccatctgtttgctctgttgaaaagaaacggaggtaacgtttccccgatgacctca 180
181 aaggatgctactggattgggatttgtcgtccgaatgtcataaagccgtacgttttgatgg 240
241 cctgctggtgccaacaactttttatcgttgggtgatttctaagcgattaacctgtgagtcg 300
301 gaatgctgaattggttcttgaacaaaccccagtaagagcctcccaaatcttattgtgtgg 360
361 tcatagccagcagataactaagataacagacatcaatcagaactcctttttataggtg 420
421 acaagactgccacggttgatcgctgctgtaataatataatgggtgtcgcactcttctataac 480
481 atgatcagtaataagctagctggttacttttctgaatcttaacgtattgtgttaccctctt 540
541 ggtttatatgaacgttccagaaacttctggaaaaagaatgggatatccttttacgggttt 600
601 ttcaaccactgtcagcacaactccttgccaaggttagttcgagatttaatatataattgta 660
661 tagatttctcgtccctttaccgtattgttaatccctttttaacttatgcttcttctgt 720
721 caaaaattctaattaatattatattaagctcttacacgaattcctttaacgacttagaatt 780
781 gtataaccatccagaaactcttcagaggataaaggataagttgtttgcattttaagattt 840
841 tttttttaatacattccacatcaatataatagagtatagtatacagaactaata ATG 896
1 M 1
897 GTC AAG GAG ACA AAA CTT TAT GAT TTA CTT GGA GTA TCT CCA AGT 941
2 V K E T K L Y D L L G V S P S 16
942 GCT AAT GAG CAA GAA CTG AAA AAG GGT TAT AGA AAA GCA GCT CTA 986
17 A N E Q E L K K G Y R K A A L 31
987 AAA TAT CAT CCA GAT AAG CCA ACA GGT GAC ACA GAA AAG TTT AAG 1031
32 K Y H P D K P T G D T E K F K 46
1032 GAG ATA TCA GAG GCC TTT GAA ATT TTA AAT GAT CCT CAA AAA AGG 1076
47 E I S E A F E I L N D P Q K R 61
1077 GAA ATA TAT GAT CAA TAC GGT CTC GAG GCT GCT AGA TCT GGT GGT 1121
62 E I Y D Q Y G L E A A R S G G 76
1122 CCA AGC TTT GGT CCT GGT GGT CCT GGC GGT GCT GGA GGT GCT GGA 1166
77 P S F G P G G P G G A G G A G G A G 91
1167 GGC TTC CCT GGC GGT GCG GGC GGA TTC TCC GGA GGA CAT GCG TTC 1211
92 G F P G G A G G F S G G H A F 106
1212 AGT AAT GAG GAT GCT TTC AAT ATT TTT TCA CAA TTC TTT GGC GGC 1256
107 S N E D A F N I F S Q F F G G 121
1257 AGT TCC CCA TTC GGT GGT GCT GAT GAC AGT GGC TTC AGT TTC TCT 1301
122 S S P F G G A D D S G F S F S 136
1302 AGT TAT CCA TCT GGC GGC GGT GCT GGT ATG GGA GGT ATG CCT GGA 1346
137 S Y P S G G G A G M G G M P G 151
1347 GGA ATG GGA GGA ATG CAT GGC GGC ATG GGA GGT ATG CCT GGC GGC 1391
152 G M G G M H G G M G G M P G G 166

| | | | | | | | | | | | | | | | | |
|------|---|-----|-----|-----|-----|-----|-----|----------------------------------|-----|-----|-----|-----|-----|-----|-----|------|
| 1392 | TTT | AGA | TCA | GCA | TCA | AGC | TCT | CCC | ACG | TAT | CCA | GAG | GAA | GAA | ACA | 1436 |
| 167 | F | R | S | A | S | S | S | P | T | Y | P | E | E | E | T | 181 |
| 1437 | GTT | CAA | GTT | AAT | TTA | CCA | GTT | AGT | CTA | GAA | GAT | TTG | TTT | GTT | GGT | 1481 |
| 182 | V | Q | V | N | L | P | V | S | L | E | D | L | F | V | G | 196 |
| 1482 | AAA | AAG | AAG | TCA | TTT | AAA | ATT | GGA | AGA | AAG | GGC | CCA | CAT | GGG | GCC | 1526 |
| 197 | K | K | K | S | F | K | I | G | R | K | G | P | H | G | A | 211 |
| 1527 | TCT | GAA | AAG | ACA | CAA | ATT | GAC | ATT | CAA | TTA | AAA | CCG | GGT | TGG | AAA | 1571 |
| 212 | S | E | K | T | Q | I | D | I | Q | L | K | P | G | W | K | 226 |
| 1572 | GCT | GGT | ACC | AAA | ATA | ACA | TAC | AAG | AAC | CAG | GGT | GAT | TAC | AAT | CCT | 1616 |
| 227 | A | G | T | K | I | T | Y | K | N | Q | G | D | Y | N | P | 241 |
| 1617 | CAA | ACG | GGC | CGT | AGA | AAG | ACT | TTG | CAG | TTT | GTC | ATC | CAG | GAA | AAG | 1661 |
| 242 | Q | T | G | R | R | K | T | L | Q | F | V | I | Q | E | K | 256 |
| 1662 | AGC | CAT | CCA | AAC | TTT | AAA | AGA | GAC | GGT | GAT | GAC | CTA | ATT | TAC | ACT | 1706 |
| 257 | S | H | P | N | F | K | R | D | G | D | D | L | I | Y | T | 271 |
| 1707 | CTG | CCA | CTA | TCT | TTC | AAG | GAA | TCA | TTG | TTA | GGT | TTT | TCA | AAA | ACT | 1751 |
| 272 | L | P | L | S | F | K | E | S | L | L | G | F | S | K | T | 286 |
| 1752 | ATC | CAA | ACA | ATT | GAT | GGC | AGA | ACC | TTA | CCT | TTG | TCG | AGA | GTA | CAG | 1796 |
| 287 | I | Q | T | I | D | G | R | T | L | P | L | S | R | V | Q | 301 |
| 1797 | CCT | GTC | CAA | CCC | TCA | CAA | ACT | TCT | ACT | TAT | CCT | GGT | CAA | GGT | ATG | 1841 |
| 302 | P | V | Q | P | S | Q | T | S | T | Y | P | G | Q | G | M | 316 |
| 1842 | CCA | ACT | CCA | AAG | AAC | CCA | TCT | CAG | AGA | GGT | AAT | TTG | ATT | GTA | AAA | 1886 |
| 317 | P | T | P | K | N | P | S | Q | R | G | N | L | I | V | K | 331 |
| 1887 | TAT | AAA | GTG | GAC | TAT | CCA | ATA | TCA | CTA | AAC | GAC | GCT | CAA | AAA | CGT | 1931 |
| 332 | Y | K | V | D | Y | P | I | S | L | N | D | A | Q | K | R | 346 |
| 1932 | GCT | ATA | GAT | GAA | AAT | TTT | TAA | tagtaatcctaagcaaatataattataaactc | | | | | | | | 1984 |
| 347 | A | I | D | E | N | F | * | | | | | | | | | 353 |
| 1985 | aaataaatttatcaccacagaaatcatataatatacatcattaattttgactcaataatc | | | | | | | | | | | | | | | 2044 |
| 2045 | ttaaattgcgcggtgcggtgcgcggttaaattaaagtttttgtatgtctgcattaccctgc | | | | | | | | | | | | | | | 2104 |
| 2105 | cctctggcatataaatgatgtatgtaaggaatggttgacttgacagaaggtcacc | | | | | | | | | | | | | | | 2159 |

Although cloning was successful and a previously sequenced gene was obtained it was difficult to ascertain whether or not the gene *SIS1* is the *SSV16* gene or a low copy suppressor.

A mapping technique employing, a set of mutants with specific unstable chromosomes, was used to identify the chromosome which carries *SSV16*. However, due to the ambiguity of testing for salt sensitivity, *SSV16* could not be pinpointed to a particular chromosome. *SIS1* resides closely linked to the centromere of Chromosome XIV and less than 1cM from *PET8* (Luke et al., 1991) .

Gene disruption, and subsequent analysis and segregation of the associated phenotypes can provide further evidence of identity of the complementing gene with the mutation. The *SIS1* gene disruption causes delayed lethality with the cells only growing for about 6 days, half the colonies being inviable and the other half producing very large cells (Luke et al., 1991). Because the null mutation is lethal its phenotype cannot be studied and in the literature there is no phenotypical analysis of point mutations which would allow us to verify any similarities between *SIS1* and *SSV16*.

In cells deleted for *SIS1* (*sis1-2*) the effects fo limited amounts of the *SIS1* protein present have been studied. When the *sis1-2* strain expressing *SIS1* under control of the *GAL1* promoter is transferred from galactose glucose media, after 31 hours the culture has an overall doubling time of 200 minutes. At this time many of the cells have become very large and many appear blocked for migration of the nucleus from mother to daughter cell. The cells also appear to have a very large vacuole with the nucleus pressed against the side of the cell (Luke et al., 1991). *ssv16-1* mutants show normal growth in a variety of media and do not show any difference in size to wild type cells as seen under phase contrast microscopy or size distribution plots in growth curve calculations using a Coulter Counter. Fluorescence microscopy of *ssv16-1* strains shows class B vacuolar morphology and normal nuclear segregation. These differences in phenotype suggest that *SSV16* is a different gene to *SIS1* and the complementation by *SIS1* may be accounted for by suppression of the *ssv16-1* mutation. However, these phenotypic differences may be accounted for by the difference in the strains studied. Work with *SIS1* was carried out using the conditional lethal strain described above whereas the study of the *ssv16-1* mutation involved the phenotypic analysis of cells containing a presumed point mutation.

SIS1 was originally isolated as a high copy suppressor of the slow growth phenotype of strains containing transcriptional suppressor mutations in *SIT4* (Arndt et al., 1989; Luke et al., 1991). *SIS1* is 1059 base pairs long encoding a 352 amino acid protein with a predicted mass of 37,592Da. It is similar (28% identical overall) to *E. coli* and *M. tuberculosis dnaJ* proteins. DnaJp is a member of the heat-shock family of proteins and has been shown to be involved in a variety of cellular processes including protein folding, proteolysis, phosphorylation and also in replication of λ and P1 phage. Sis1p is most similar in the amino-terminal third of the protein (50% identical for *SIS1* and *E. coli dnaJ*

for the first 104 amino acids). There is also similarity in the terminal third. The middle third (100-200) has very little similarity to *dnaJ* which contains 4 cysteine rich repeats which are not present in Sis1p which has a glycine/methionine rich sequence. *SIS1* is heat-shock inducible (Luke et al., 1991).

SIS1 has similarity to other *dnaJ* homologues identified in yeast. The *SEC63* protein which is required for import of proteins into the ER contains a 70 amino acid region that is similar to the amino-terminal 70 amino acids of *SIS1* or bacterial *dnaJ* proteins (Sadler et al., 1989). It is also similar to *YDJ1* a yeast protein isolated by using antibodies directed against matrix lamina pore complexes (Caplan and Douglas, 1991) and *SCJ1* protein which on overexpression causes missorting of a nuclear-targeted test protein (Blumberg and Silver, 1991).

Sis1p, Sec63p and Ydj1p have all been located to the nucleus or ER. This suggests another point in favour of *SIS1* acting as a suppressor of *ssv16-1* which is possibly involved in protein targeting between the Golgi apparatus and vacuole, which would suggest that the *SSV16* protein would be associated with these organelles. The association of Sis1p with the endomembrane system may in some unknown way be able to compensate for the lack of Ssv16p at a later stage of the secretory pathway.

Much work needs to be done to discover the relationship between *SSV16* and *SIS1*. The main need is to isolate the *SSV16* gene thereby proving conclusively that *SIS1* and *SSV16* are two separate genes. A number of methods may be tried. *ssv16-1* strains transform very efficiently and so further attempts at cloning by complementation may be tried implementing a new library. The large number of colonies transformed without producing a positive clone may be caused by under-representation of the genome due to amplification of the existing plasmid library. Furthermore, once *SSV16* has been designated to a specific chromosome, its map position on the chromosome can be found by meiotic mapping revealing linkage to other known chromosome specific markers. Overlapping cosmid clones of the chromosomes are available and cloning by subsequent complementation of a subset of these clones may help to isolate the gene.

CHAPTER 5

CHARACTERISATION OF MLY0508

5.1. Introduction.

MLY0508 was isolated by mutagenesis of the wild type stain SEY6211. Selection for impaired growth on media supplemented with 1.5M NaCl was made after seven days of incubation at 30°C. Mutations were designated *ssv* for salt sensitive vacuolar. MLY0508 was placed in complementation group *ssv17*. The 23 mutants in this group showed only weak complementation with a large proportion of the other *ssv* mutants thus exhibiting a semi-dominant phenotype. However the 23 mutants within this complementation group are all phenotypically similar and completely fail to complement each other (Latterich, 1992). MLY0508 was designated *ssv17-101*.

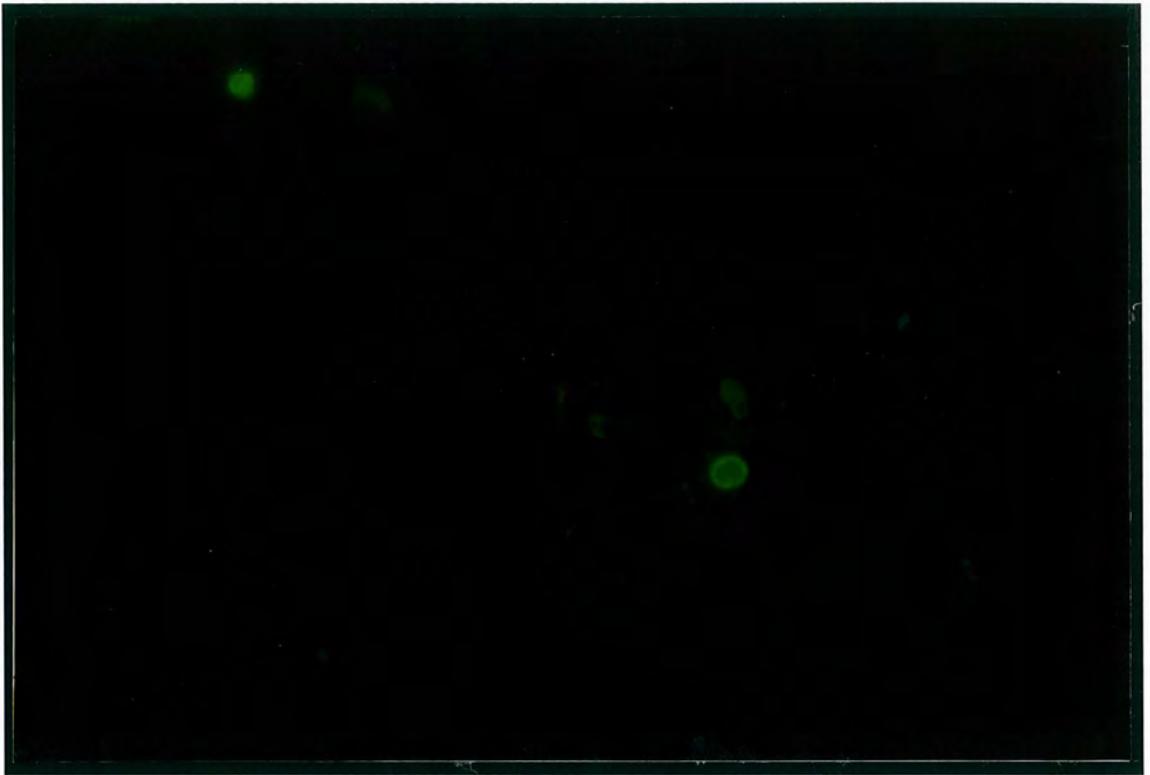
Microscopic techniques were used to study the vacuolar morphology of MLY0508. Normarski contrast optics suggested that vacuoles belonged to class A/C whereas electron microscopy, using a preparation method specifically staining double membranes and carbohydrate structures that accentuate vacuoles, indicated class C vacuolar morphology (Latterich, 1992). MLY0508 also exhibits a Pet^- phenotype being unable to grow on a non-fermentable carbon source such as glycerol, this has also been observed in a number of *vps* mutants (Raymond et al., 1992). MLY0508 was tested for secretion of various vacuolar proteinases and showed significant secretion of PrA, PrB and CPY in a non-lysis colony immunoblot.

MLY0508 was chosen for further study to try to elucidate the features of the *ssv17* complementation group. It was hoped that cloning of the gene and analysis of the protein involved would help to unravel some of the interesting characteristics and see how some of these functions interact. Work described in this chapter indicate that MLY0508 belongs to a small group of vacuolar segregation defective mutants and attempts at cloning the *ssv17-101* gene have been described.

Figure 5.1. Immunofluorescence of *ssv17-101*.

Picture A shows cells stained with anti-Pho8p and visualised under FITC fluorescence. This illuminates the vacuole. Picture B shows the corresponding DAPI stain as visualised under UV illuminating and highlighting the nuclei. The photographs clearly show the vacuolar segregation defect exhibited by *ssv17-101*. Note the large budding cell in the centre in which the maternal cell contains a normal vacuole but the bud appears to have no vacuole. However the DAPI staining shows both cells contain nuclei.

A.



B.



5.2. Results.

5.2.1. Indirect Immunofluorescence.

Indirect immunofluorescence using antibodies to two vacuolar membrane proteins, Pho8p and the 60KkDa subunit of the vacuolar ATPase allows the study of vacuole morphology in whole cells. The results with MLY0508 showed a mixed morphology with some cells exhibiting classic A (normal) type vacuoles and others with apparently no vacuoles. A study of 100 cells suggested that *ssv17-101* mutants exhibited a vacuolar segregation defect. This is typified by the presence of a large bud with no apparent vacuole budding from a maternal cell with a class A vacuole (figure 5.1.). Segregation of the vacuole precedes nuclear segregation transfer but numerous budding cells exhibited this vacuole segregation defect late into cell division when nuclei, illuminated by DAPI staining, could be seen in both cells. Both cells in figure 5.1. showed DAPI staining indicating the presence of a nucleus.

5.2.2. Transformations.

MLY0508 was transformed with the YCp50 based gene library provided by M. Rose (Rose et al., 1987) using electroporation (section 2.9.3.). Transformation was undertaken on three separate occasions, from a total of ten independent transformations 5-15 Ura⁺ colonies per plate were recorded. Low transformation frequency resulted in the implementation of three other methods to try to improve efficiency. One method employed electroporation but with a modified cell preparation procedure devised by M. Latterich using 10mM Tris HCl pH7.0, 270mM sucrose and 1mM MgCl₂ as the buffer in which cells were washed and resuspended before electroporation (Latterich, 1992). A second method employed Lithium acetate to produce competent cells (Latterich, 1992). These methods repeated on two separate occasions failed to produce any transformants.

The third method involved the use of a *kar1-1* (DBY1710) strain to act as a plasmid carrier. Unlike MLY0508, the *kar1-1* strain transforms efficiently and due to its nuclear fusion defect, a transformed strain of *kar1-1* is able to pass on the plasmid to another cell during mating without fusion of the nuclei or mixing of genetic material. The method involves transformation of a *kar1-1 ura3* strain with the YCp50 based library and selection for Ura⁺ transformants. These colonies are crossed to a *ssv17-101 ura3 can^R* strain by replica plating the Ura⁺ transformants onto a lawn of the recipient strain. 'Cytoconductants', cells that have received the plasmid but not the nucleus from the transformed *Kar1-1* strain, were selected for on plates lacking uridine but containing

canavanine (at 50 µg/ml) taking advantage that the drug resistance is a recessive trait. This protocol was devised in the laboratory of David Botstein.

MEY0508-26 was backcrossed to AB1380 and a strain selected with salt sensitivity, Ura⁻ and canavanine resistance (CHY1001). The library was transformed into DBY1710 using electroporation (section 2.9.3.). The recipient CHY1001 was grown overnight in 10ml YPDA then 200µl spread onto a YPDA plate and allowed to dry. The transformed DBY1710 cells were replica plated onto the recipient strain and incubated overnight at 30°C. These were subsequently replica plated onto YMM -Ura +canavanine (80µg/ml) to select for cytoconductants which were subsequently tested for salt sensitivity by streaking onto YPDA+1.5M NaCl.

The transfer of the library plasmid from DBY1710 to CHY1001 was very poor resulting in less than 50% transfer. The initial study produced 1456 cytoconductants which failed to grow on salt plates. DBY1710 was re-transformed with the library and 4900 transformants isolated. 500µl of CHY1001 was added to the YPDA plates to ensure an evenly spread lawn, however the percentage transfer was still less than 50%. Most of the colonies grew on salt plates and were purified on salt supplemented media. Plasmids from ten colonies were isolated and identified as YCp50.

The library used was transformed into *E. coli* DH5α and 20 plasmids isolated and cut with *Hind*III. The results showed that the library used contained less than 50% YCp50 with an insert.

Due to the poor transformation of MLY0508, the dubious quality of the library and the difficulty surrounding use of the *kar1-1* strain as a plasmid transfer system, cloning by complementation was rejected in favour of a chromosome mapping method.

5.2.3. Chromosome Mapping.

MEY0508 was crossed to each of 16 "B-strains". These strains are cir⁰ containing the 2µ plasmid FLP region inserted into a specific chromosome. When crossed to a cir⁺ strain they lose a single specific chromosome during replication in a glucose rich media (YPDA). Diploids were isolated on YMM-lysine and methionine. The 16 diploids were cultured twice in 10ml YPDA liquid before being diluted and plated onto YPDA plates. 50 colonies from each strain were streaked onto 1.5M salt plates. Their growth was recorded according to the number of colonies that did not grow on salt plates due to the *ssv17-101* gene being uncovered by the loss of a chromosome (Table 5.1.). Results show that loss of chromosomes III, IV, V, VI, VII, XIII, XVI do not uncover the *ssv17-101* mutation. One colony failed to grow due to the loss of chromosomes I, VIII and XII. Two colonies

| Strain | Chromosome | Experiment 1 |
|--------|------------|--------------|
| B7588 | I | 1 |
| B7170 | II | 2 |
| B7171 | III | 0 |
| B7589 | IV | 0 |
| B7590 | V | 0 |
| B7591 | VI | 0 |
| B7173 | VII | 0 |
| B7174 | VIII | 1 |
| B7175 | IX | 2 |
| B7593 | X | 2 |
| B7178 | XI | 3 |
| B7595 | XII | 1 |
| B7255 | XIII | 0 |
| B7596 | XIV | 7 |
| B7180 | XV | 4 |
| B7598 | XVI | 0 |

Table 5.1. Chromosome loss.

Loss of a specific chromosome from diploids constructed from MEY0508-26 and the B-strains results in expression of the *ssv* phenotype which can be detected by failure to grow on salt plates. Column one lists the 16 B-strains which were backcrossed to *ssv17-101*. The chromosome number in the second column indicates the one which is lost on subsequent division. The third column tables the results of growing the diploids twice in YPDA during which time a specific chromosome is lost and the number of colonies which show salt sensitivity when 50 colonies are plated onto salt plates.

exhibited salt sensitivity due to the loss of chromosomes II, IX and X and the loss of chromosomes XI, XV and XIV resulted in 3, 4 and 7 colonies respectively failing to grow.

Many *yps* mutants exhibit a *Pet*⁻ phenotype, failing to grow on media with glycerol as the carbon source, *ssv17-101* also exhibits this phenotype. Therefore five diploids were re-tested and the cells plated onto YPGA plates (substituting 2% glycerol for glucose) to test for growth. The results of the chromosome loss experiments on diploids of B7588 (I), B7170 (II), B7255 (XIII), B7596 (XIV) and B7180 (XV) are shown in table 5.2..

| Strains | <u>colonies showing growth</u> number of colonies tested |
|---------|---|
| B7588 | 500/500 |
| B7170 | 1399/1400 |
| B7255 | 700/700 |
| B7956 | 0/500 |
| B7180 | 500/500 |

Table 5.2. Expression of *ssv17-101* *Pet*⁻ phenotype due to chromosome loss.

Another 1000 colonies were tested of strains B7170 and B7596 on glycerol plates, this showed all the B7170 colonies grew on glycerol but none from B7596 grew. It was concluded that *SSV17* resided on chromosome XIV due to the manifestation of the recessive salt sensitive or *Pet*⁻ phenotype on loss of chromosome XIV present in the MEY0508-26/B7596 diploid.

5.2.4. Meiotic Mapping.

In order to determine where on chromosome XIV the *SSV17* gene resided, meiotic mapping was carried out. This involves crossing the unknown gene with strains carrying mutations whose position has been mapped on a particular chromosome. After sporulation of the heterozygous diploids, tetrads are dissected and the individual spores analysed for segregation of the various markers. There are three types of tetrads from a hybrid which is heterozygous for two markers AB x ab: PD (parental ditype), NPD (non-parental ditype) and T (tetratype). By studying the ratios of these three tetrad types isolated from a spore dissection, gene and centromere linkage can be deduced (table 5.3.).

Random assortment occurs when either two genes are widely separated on the same chromosome or if the two genes are on different chromosomes and at least one is not centromere linked. If the two genes are linked there is an excess of PD to NPD. If two

genes are on different chromosomes and are linked to their respective centromeres there is a reduction in the proportion of T-asci.

| | PD | NPD | T | | |
|--------------------|----|-----|----|---|----|
| | AB | Ab | AB | | |
| | AB | Ab | Ab | | |
| | ab | aB | aB | | |
| | ab | aB | ab | | |
| Random Assortment | 1 | : | 1 | : | 4 |
| Linkage | >1 | : | <1 | | |
| Centromere Linkage | 1 | : | 1 | : | <4 |

Table 5.3. Tetrad analysis.

Initially MLY0508 was crossed with XS1-3A this strain contains two chromosome XIV markers on different arms of the chromosome, *met2* is on XIVL and *lys9* is on XIVR. However, after two separate attempts the diploid would not sporulate. Consequently MEY0508-26 was crossed to strain 368, this contains *rna2-1* on the R arm of the chromosome approximately 8cM from the centromere. Further information (see next section) indicated that a *VAC1* homologue was present on chromosome XIVR closely linked to the centromere. Strains with *vac1* mutations also exhibit a vacuolar segregation defect, and it was proposed that this *VAC1* homologue could be *SSV17*. If this is true then tight linkage would be expected between the *ssv17-101* salt sensitive phenotype and *rna2-1*.

Spores were dissected and grown on YPDA media then replica plated onto YPDA + 1.5M NaCl and incubated at 30°C or onto YPDA and incubated at 39°C. Tetrads were scored according to their growth on both media. Sporulation was successful and 79 spores were dissected. The results were inconclusive (table 5.4.).

| Tetrad types | PD | NPD | T |
|-------------------|--------------|---------|---------|
| | rna SSV | rna ssv | rna SSV |
| | rna SSV | rna ssv | rna ssv |
| | RNA ssv | RNA SSV | RNA ssv |
| | RNA ssv | RNA SSV | RNA SSV |
| Number of tetrads | 3 | 17 | 41 |
| | Inconclusive | 18 | |

Table 5.4. Tetrad analysis of the MLY0508/XS1-3A diploid.

18 colonies could not be categorised and were the result of poor spore dissection. The ratio of 1: 6: 14 (numbers rounded up) was observed which suggests that no linkage occurs between *ssv17-101* and *rna2-1*.

5.2.5. PCR.

Sequencing of Chromosome XIV as part of the EC Yeast Genome Project indicated a *VAC1* homologue on chromosome XIV, closely linked to *PET8* and the centromere (Verhasselt and Volckaert personal communication). Due to the Vac phenotype of *ssv17-101* it is possible that *SSV17* and the *VAC1* homologue ("VAC1H") are identical. It was decided to check VAC1H for complementation of *ssv17-101*.

Information concerning gene size, restriction enzyme data and sequence data from either end of the gene, supplied by P. Verhasselt, allowed cloning of VAC1H by PCR. From the sequence data (figure 5.2) two 22mer primers were produced.

| | |
|------------|-------------------------------|
| Primer 985 | CTA TTA GCT GCA AGC CAG GTG G |
| Primer 986 | CTC ATC TAA TAT GCA CGG AGC C |

The position of the primers in relation to the VAC1H is shown in figure 5.3.. A PCR reaction was set up as in Materials and Methods using genomic DNA extracted from wild type cells (section 2.6.6.). 10µl of the total 100µl reaction was electrophoresed on an agarose gel showing a distinct band at the expected fragment size, 2.7kb (figure 5.4.).

Primer 985

GACGGTGTCCGAGACTGGGAGGATTTACAAGACGATATTGATGATTCGTTACGGGTAGACCA
ATACTTGATTGTTTTCTTAAAGTTTATTACTAGTGTTACACCGAGTATAACAACATGACTATA
CCATGGAAGGTTGGTAAAAATTTGTGTTTTTTCA **ctattagctgcaagccaggtgg** TCAA
AAAACAGGATAAACAAGAAGATAGGAATTAATAAATATGCAGGAACAAGTTTCTTCCTT
AAAACCACTTTCTTAAATGGTCCT

Primer 986

TTGTCTAGTACATAGTTACTGGTGAACTTTCCTGGATATGCGCGTGCTAACTAGAGCATTTT
GTGTTGATAATTAATTTAAATTGCATGATACAGATTACGAGACGAAAGATAGTCGATTATAT
ATCTTTCAATCTTGGTTTAGTTTGGCAGCAAAAGCTAAAAGGTA **ggctccgtgcatattag**
atgag TGAACACACTTTAGGTTTATTCTTGTATGAAATCATTCACTTCGACCTCTTCCAAG
ATCAAATTCTCTAATGGATTACTTATGAGTTTACCAAATTCGTCAAATTCCAACCTGACTAC
ATTCAAATTCTTAAC

Figure 5.2. Primer data.

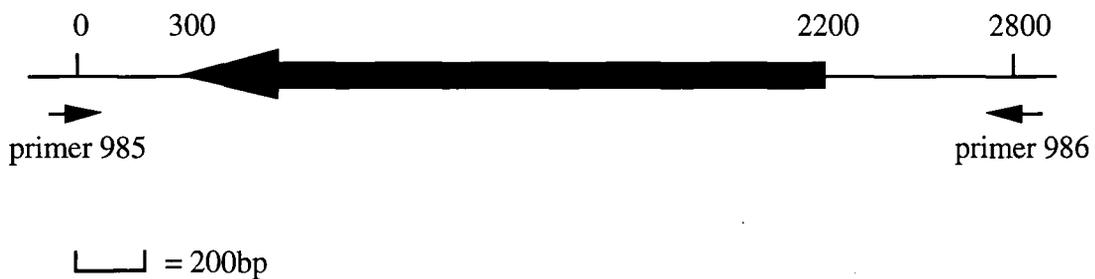
271 base pairs around the 5' end of the Vac1 homologue and 323 base pairs around the 3' end were provided by P. Verhasselt. From this information a stretch of 22 bases were chosen and primers made. The primers are denoted by a stretch of bold, lowercase letters. The 5' primer is situated approximately 300bp from the end of the open reading frame. The 3' primer is approximately 600bp from the start of the open reading frame.

Figure 5.3. Restriction enzyme map and positioning of primers of Vac1 homologue.

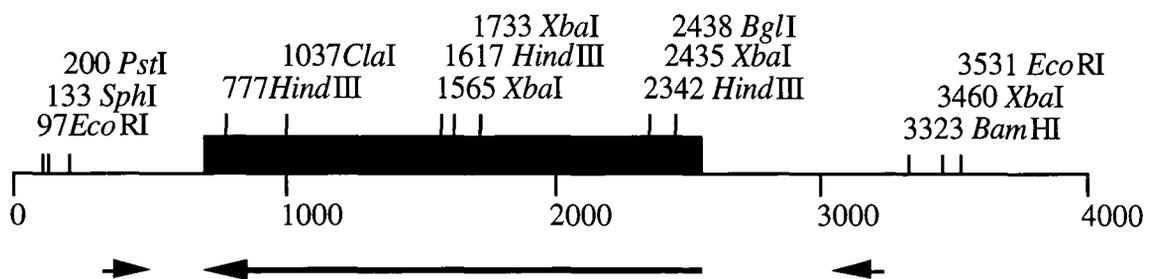
Fig. 5.3.A. shows the positions of the two primers relative to the Vac1 homologue. The open reading frame is approximately 1.9kb and was amplified by PCR from genomic DNA within a 2.8kb fragment using the primers indicated.

Fig. 5.3.B. shows the digest pattern for eight restriction enzymes along a 4000bp stretch of DNA. The black box represents the Vac1 homologue the arrow below this box shows the direction of translation. The two smaller arrows show the approximate positions of the primers.

A.



B.



The fragment was isolated from an agarose gel using silica fines before being ligated into pUC19. The lack of any appropriate enzyme sites within the primers led to the ligation of the fragment into a blunt ended site (*HindII*) in pUC19. 3'-recessed termini in the PCR product were filled using Klenow polymerase replacing the 10x Klenow buffer with the appropriate amount of 5x ligation buffer, after 30 minutes at room temperature the enzyme was denatured at 70°C for five minutes before cooling on ice. To this reaction 2µl of pUC19 cut with *HindII* and 1µl of ligase were added and the ligation mixture incubated overnight at 4°C. 10µl of this mixture was transformed into DH5α and plasmids prepared from transformed cells by the silica fines method. Two plasmids were isolated which contained the fragment in pUC19. Preparation of pure plasmid DNA by alkaline lysis with a PEG precipitation step was done and the DNA sequenced using standard forward and reverse primers (figure 5.5.). Data corresponded to the sequence surrounding the PCR primers provided by P. Verhasselt (figure 5.3.) and it was concluded that the *VAC1* homologue had been cloned.

5.2.6. Expression of Vac1 homologue in the yeast centromeric plasmid YCp50.

In order to express the *VAC1* homologue in MLY0508 it was inserted into a yeast centromeric vector, YCp50. The pUC19+ PCR product (pCH40) was digested with *SphI* and *BamHI* in order to isolate the insert. pUC19 and the insert are both approximately 2.7kb in size so the single band from this digest was excised from an agarose gel. The DNA isolated and digested with *ScaI* which has a single site in pUC19. Three bands were produced at 2.7kb, 1.7kb and 960bp, this corresponded to the insert and two pUC19 fragments respectively (figure 5.6.). The insert was isolated from the gel and three attempts were made to clone it into *SphI/BamHI* cut YCp50. Subcloning into the yeast vector was unsuccessful (see Discussion)

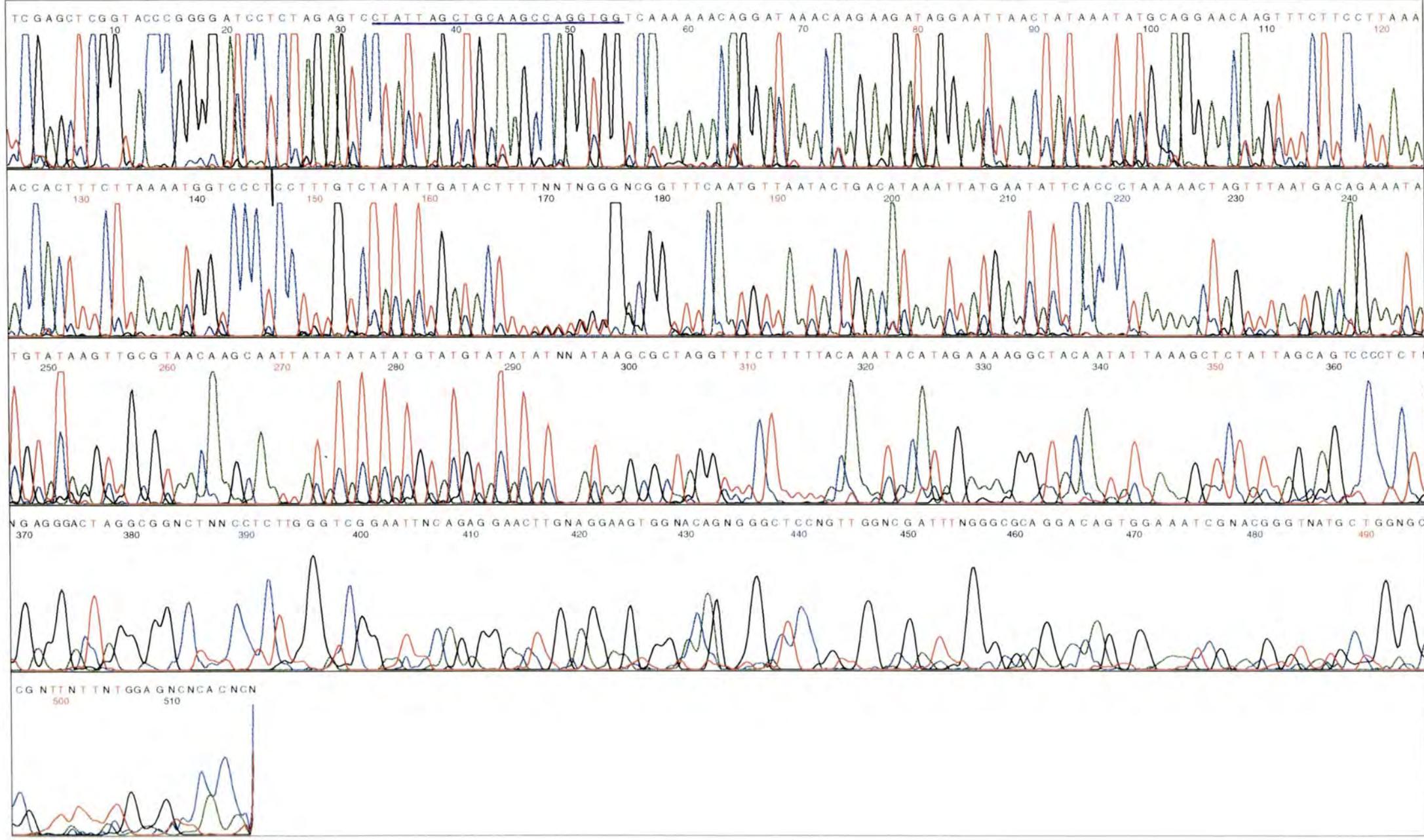


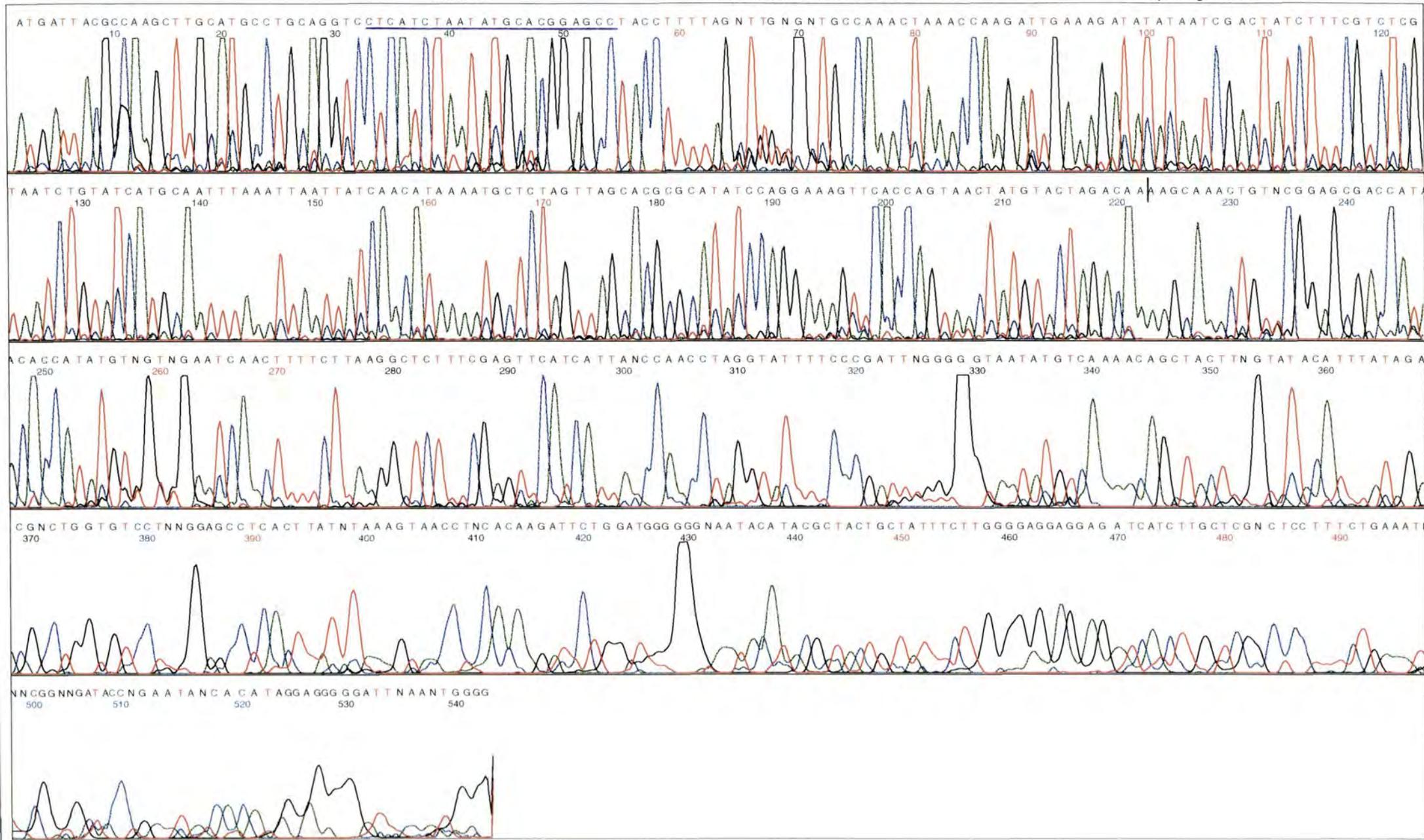
Figure 5.4. PCR of Vac1 homologue.

Lane 1 shows the λ *Pst*I ladder. Lanes 2 shows a 10 μ l sample from a PCR reaction using genomic DNA as the template and 16 μ l of MgCl₂ in the reaction mix. Lanes 3 and 4 show a PCR amplification of previously isolated Vac1 PCR product using 32 μ l and 16 μ l MgCl₂ respectively in the reaction mix. The larger band is found between the 2.49 and 2.84kb bands of the λ ladder. This corresponds to the predicted size of the PCR product. Further experiments used the product in lane 2

Figure 5.5. Sequencing of pCH40.

The pUC19 + PCR insert (pCH40) was sequenced automatically using the two 22mer primers. Data corresponded to the sequence provided around the primer sites (figure 5.2.). The first printout shows the forward sequence using primer 985 and the second is that of the reverse primer 986. Primers are underlined in blue and the end of the verified sequence is shown by a vertical black line.





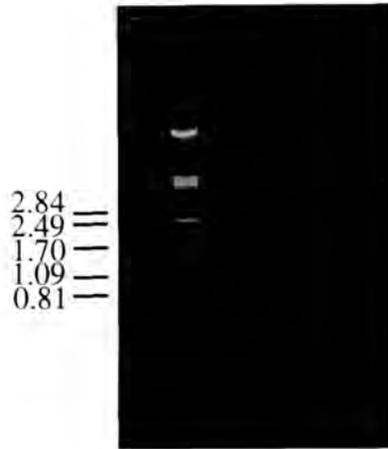


Figure 5.6. Isolation of the *Vac1* homologue from pCH40.

pCH40 was digested with *SphI*, *BamHI* and *ScaI* in order to recover the *Vac1* homologue to be cloned into a yeast vector. Three bands are clearly visible at approximately 2.7kb, 1.7kb and 960bp. Arrows indicate the positions of fragments of known size on the λ Pst marker.

5.3. Discussion.

The *ssv17-101* mutation confers a number of phenotypes affecting a variety of cellular processes: osmoregulation, protein sorting and vacuole segregation. Isolated as a salt sensitive mutant with a potential role in osmohomeostasis, further characterisation was undertaken in order to elucidate the role of the vacuole in a number of pleiotropic events. Initial characterisation has revealed secretion of large quantities of vacuolar proteinases similar to the *vps* mutants. One feature of note is its partial complementation of the other 16 *ssv* groups although it fails to complement the other 23 alleles in the *ssv17* group (Latterich, 1992). Complementation was determined by the ability to grow on high osmotic strength media.

Investigation into the vacuolar morphology using fluorescence microscopy techniques revealed that *ssv17-101* also belongs to the relatively small group of vacuolar segregation mutants. These mutants form a large bud which fails to inherit vacuolar material. In wild type cells the process of vacuole inheritance precedes that of nuclear segregation (figure 5.7.). As soon as the bud emerges it acquires a vacuole the volume of which increases as the bud grows (Weisman et al., 1987). The emergence of this vacuole is partly due to the partitioning of the vacuole from the mother to the daughter along tubulovesicular structures (Weisman et al., 1990). The process is completed by the onset of nuclear migration (figure 5.7.). Vacuoles of MLY0508 were illuminated showing some budding cells in which the mother contained a vacuole but the daughter did not. Both mother and daughter cells exhibited nuclei, therefore *ssv17-101* was classified as a vacuole segregation mutation.

Other vacuole segregation (*vac*) mutants were isolated by screening *vps*-like mutants for those exhibiting temperature sensitive growth and vacuole segregation defects. Of the mutants isolated two strains have been characterised. *vac1* mutants secrete CPY and are temperature sensitive (Weisman et al., 1990; Weisman and Wickner, 1988) *vac2* mutations are temperature sensitive but do not show any *vps* characteristics. Neither strain exhibits salt sensitivity and were presumed to be different to *ssv17-101*. Testing for complementation between the *vac* mutants and *ssv17-101* would substantiate claims that *ssv17-101* is not a previously identified *vac* strain. This is supported by the clear phenotypic differences between *vac2* and *ssv17* strains. All other *vac* mutations are new alleles of previously identified *VPS* genes. It is known that *SSV17* is not identical to any *VPS* gene (M.D. Watson, T. Stevens personal communication). Therefore this was considered sufficient grounds to proceed with the cloning of *SSV17*.

The difficulties experienced in transformation of MLY0508 proved cloning by complementation with a gene library unviable and so efforts were concentrated on classical mapping techniques. Chromosome mapping used a set of 16 *cir*⁰ tester strains which due to the integration of a 2 μ inverted repeat sequence near the centromere results

in the loss of a specific chromosome in diploids, therefore resulting in the expression of the unmarked recessive mutant phenotype. This method proved successful and *ssv17-101* was mapped to chromosome XIV. Gene mapping did not prove so successful and results produced no conclusive evidence of linkage to the *rna2-1* gene which is situated close to the centromere on chromosome XIV.

Information from P. Verhasselt suggested a *VAC1* homologue was present on chromosome XIV closely mapping to the centromere. This gene was isolated by PCR techniques. Attempts were made to clone the *VAC1* homologue into a yeast vector in order to allow its introduction into MLY0508 to test for complementation of the *ssv17-101* mutation. A positive identification of the *ssv17-101* gene would allow further study into its role in osmoregulation, vacuole segregation and protein sorting mechanisms.

If the PCR product does not complement MLY0508 then detailed work may be undertaken to identify the position of *ssv17-101* on chromosome XIV. This may be done using the method of meiotic mapping already described or by the use of complementation by cosmids covering the whole of chromosome XIV.

Experiments subsequent to those described in this chapter indicate that the *VAC1* homologue does not complement *ssv17-101*.

Figure 5.7. Vacuole and nucleus segregation in wild type yeast cells.

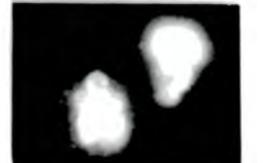
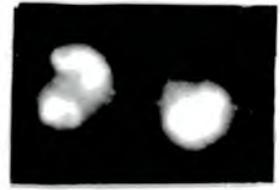
Early in the onset of budding part of the maternal vacuole segregates to the daughter bud. The figure shows the progression of this segregation with relation to the cell cycle and nuclear segregation.

The first column shows the growing bud. The second column highlights the progress of nuclear segregation during budding and the third column depicts the corresponding vacuole fluorescence, illuminating segregation of the vacuole. Note that early in budding the vacuole segregates to the daughter bud, this is followed by partitioning of the nucleus.

Normarski

DAPI

FITC (anti-pho8)



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