The expression of plant vicilin DNA in yeast

Briggs, Leslie

THE EXPRESSION OF PLANT VICILIN DNA IN YEAST.

Submitted by :-

LESLIE BRIGGS B.Sc. (Hons)

Manchester Polytechnic.

Dissertion submitted in partial fulfilment of the requirements for the degree of Master of Science, University of Durham.

Department of Botany. September, 1986.

ABSTRACT.

The aim of this project was to study the expression of recombinant DNA plasmids containing the plant seed protein gene for vicilin, in the yeast *Saccharomyces cerevisiae*. The fidelity of expression was detected by a complete functional protein as judged by binding to anti-vicilin anti-body. This allowed one to gain an insight into the molecular genetics of yeast, especially for expressing heterologous plant proteins. The aim can be further subdivided into a variety of studies; (i) the expression of a genomic vicilin clone, containing the introns and using a yeast promoter, in the yeast, (ii) the expression of a cDNA vicilin clone, which contains no leader sequences, (iii) a study of the transformation systems used for yeast, and (iv) the analysis of the expression using electronmicroscopy, and the study of the secretory pathway involved.

The results obtained in this project can be elucidated in the relevent sections of the text. But the production of the genomic clone had to be abandoned as obtaining the genomic DNA proved to be too difficult, as the time allocated to the project was limited. The cDNA vicilin plasmid was constructed, but again due to the limited time, the plasmid could not be transformed into the yeast and analysed. The study of the transformation systems proved to be successful, except the genomic and cDNA clones were not studied. Using the electronmicroscope, the micrographs obtained were only of the yeast containing the plasmid pDUB2018, which contains the same cDNA sequences used in (ii).

These showed that the leaderless cDNA was expressed and could be seen to be partially associated with membrane structures.

DECLARATION.

No part of this work has been submitted by me for any degree at this or any other University. All the work presented was done by me, except where otherwise stated in the text.

CONTENTS.

LIST of FIGURES.

LIST of TABLES.

ACKNOWLEDGEMENTS.

I would like to thank all the members of the Botany Department for making the time spent there so enjoyable. I thank Dr. Martin D. Watson for first generating my interest in the project, his help and guidance throughout this work. I would also like to thank the technicins Mr. Alan Duffall and Miss Helen Grindley for their help in the project. Finally I would like to thank my parents, without their support this Year would not have been possible.

ABBREVIATIONS.

The following abbreviations were used:

Alec's gel buffer	=	40mM Tris-acetic acid pH7.7, 2mM EDTA
ATP	=	Adenosine triphosphate.
bp	=	base pairs.
BSA	=	Bovine Serum Albumin.
cDNA	=	complementary/copy DNA
CTP	=	cytidine triphosphate.
d	=	deoxy-
dd	=	dideoxy-
DMF	=	dimethylformamide.
DMSO	=	Deoxy-ribonucleic acid.
ds	=	double-stranded.
DTT	=	dithiothreitol.
EDTA	=	ethylene diamine tetra-acetic acid.
GTP	=	guanosine triphosphate.
HC1	=	Hydrochloric acid.
H.p.l.c	=	High performance liquid chromatography.
HPLC grade	=	Double distilled and deionised.
IPTG	=	Isopropyl-β-D-thiogalactopyranoside.
kb	=	Kilo base pairs.
lac	=	β-galactosidase gene system.
Miniprep	=	Miniature preparation.
Minipreped	=	Prepared by the miniprep method.

Abbreviations contd/...

Mr	=	Molecular Weight.
mRNA	=	messenger RNA.
n	=	nano.
O.D.	=	Optical Density.
PEG60000	=	Polyethylene glycol 6000
Poly A	=	Polyadenylic acid.
Poly(A)	=	Retained by an oligo (dT) cellulose column.
RNA	=	ribonucleic acid.
RNAsein	=	RNAase inhibitor.
S	=	Svedburgs.
ss	=	single-stranded.
SDS	=	Sodium Dodecyl-sulphate.
SSC	=	0.15 sodium chloride,15mM sodium citrate to pH 7.0 with sodium hydroxide.
μ	=	mu
⇒	=	to

CHAPTER 1

INTRODUCTION

1. INTRODUCTION:

Yeasts are eukaryotic microbes whose cells, despite their small sizes, have a complex cytoplasmic organisation similar to that of higher eukaryotes, and therefore serve as useful tools for the molecular analysis of the eukaryotic system. The particular advantages of yeast cells have been outlined many times [38,44,70], namely; ease of handling; rapid growth in a genetically homogeneous population; control in alteration between haplo- and diplo-phases; well-established isolation of cellular components and organelles (nuclei, mitochondria, vacuoles); isolation of diverse mutants; powerful methods of genetic, biochemical, and cytological analysis and most importantly in the view of this project, the utilisation of recombinant DNA and protoplast fusion techniques. Yeast offers a 'simple' system in which to study molecular processes, and by doing so a greater understanding of more complex eukaryotic systems can be determined.

Yeast shares with both animal and plant cells many of the molecular processes that are currently of interest to cell biologists. It has, for example, ubiquitin, calmodulin, clathrin, endocytosis, peptide growth factors, small nuclear RNAs, retroviral elements, and actin and tubulin. Thus, such central phenomena as protein localisation (secretion), function and synthesis of the mitotic apparatus, and the workings of the cell cycle can all be productively studied in yeast. About any biochemical, cytological or genetic technique or experimental strategy can be applied in studies with yeast. Indeed, many aspects of protein and compartmentalisation appear to employ

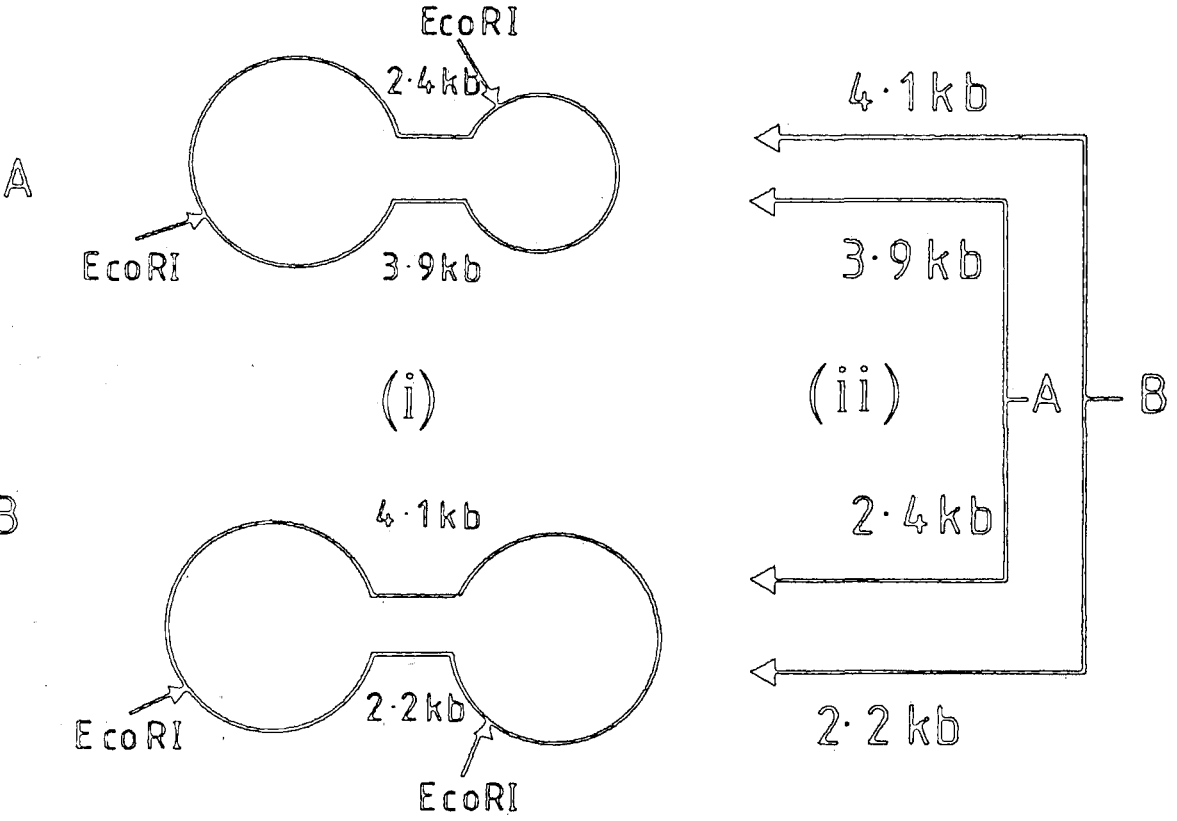
uniform mechanisms in mammals, plants and yeast [25]. It is the possession of vacuoles and their possible functional similarity with plant vacuoles that prompted this project; expression of cDNAs from the plant vacuolar protein Vicilin in yeast and its subsequent posttranslational modification and sequestration.

1.2 Yeast DNA:

The genome size of the well studied budding yeast *Saccharomyces cerevisiae* is as small as 1.0 x10^{10} daltons per haploid cell [38,74], only fourfold that of *Esherichia coli* genome, and can be pooled into 4600 colonies of *E.coli* as DNA fragments of average 10 x 10^{6} daltons [28,38,106]. About 80% of the total genome resides in chromosomal DNA. The detailed genetic map is constructed in 17 chromosomes on which more than 300 genes have been located[38,106]. The fission yeast *Schizosaccharomyces pombe*, whose genome size is comparable to that of *S.cerevisiae*, has three large chromosomes with approximately 70 genes mapped on them [38,54,96,97].

Yeast are ideal candidates for genetic manipulation since they are uninucleate cells that posses a wealth of extrachromosomal genes or genetic factors. The question of the existence of genes in the cytoplasm was first approached by the pioneering work of Ephrussi and his co-workers (1952, 1955)[28,38,96,97], which showed that the inheritance of a mitochodrial petite mutation of *S. cerevisiae* follows non-mendelian law.

FIG. 1.1


EcoRI
2·4kb
EcoRI
3·9kb
(i)
4·1kb
3·9kb
(ii)
A
B
2·4kb
2·2kb
4·1kb
EcoRI
2·2kb
EcoRI
(a)

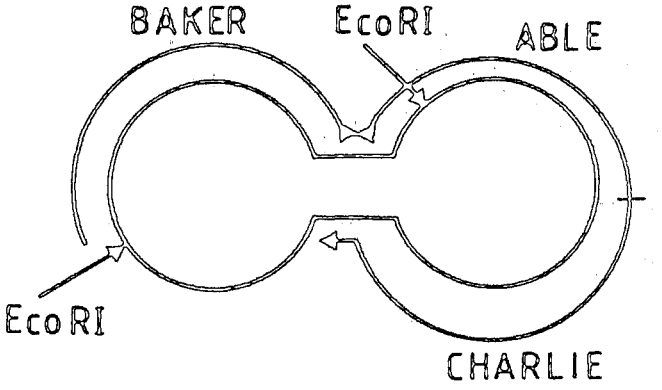

BAKER
EcoRI
ABLE
EcoRI
CHARLIE
(b)

FIGURE 1.1

(a) Physical maps of the two forms of the 2μm DNA with EcoRI cleavage sites.

(i) the two forms (A and B) of the 2μm DNA in the dumbell representation to indicate the positions of the inverted repeat sequences (stem of dumbell). the lengths of the EcoRI digestion fragments are indicated.

(ii) A diagrammatic representation of the banding pattern of fragments of EcoRI-digested 2μ DNA after electrophoresis through agarose. The pairs of DNA fragments derived from form A or form B plasmid molecules are indicated.

(b) The locations of translation ORFs in 2μ DNA. Type A 2μ plasmid DNA is shown in the dumbell representation, indicating the locations of the translation ORFs ABLE, BAKER, and CHARLIE as determined by Hartley and Donelson (1980)[106]. Able is encoded by the DNA strand of opposite polarity to Charlie and Baker as indicated by the arrows.

1.3 Yeast DNA plasmids:

Yeast cells harbour a 2μm DNA of unknown function in 25 to 100 copies per cell, amounting to about 4% of the total DNA. The plasmid 2μm DNA is a class of covalently closed circular DNA (cccDNA) with a monomeric contour length of approximately 2μm [14,38,106]. Most strains of *S. cerevisiae* contain the plasmid, which is also found in *S. italicus*, *S. diastaticus*, *Torulopsis dattila*, and several other brewery yeast [14,38,106]. The basic structural features were first demonstrated by electron microscopic analysis. The entire sequence consists of 6318 base pairs(bp), which are separated by identical inverted repeats of 599 bp into two unique sequences of 2774 bp (L region) and 2346 bp (S region). There are three long, open reading frames (ORF), "ABLE" and "CHARLIE" on the S region and "BAKER" on the L region, each of which partly extend into the inverted repeat regions (figure 1.1B). The 2μ plasmid-mediated production of polypeptides in yeast [38,74,96,106] and in *E. coli* [35,38], has been reported, although the biological role of the plasmid is yet unknown.

The 2μm DNA exists in yeast cells in a mixture of two isomeric forms distinguishable from each other by the relative orientation of their L and S regions [14,38,96,97,106]. The isomers (figure 1.1A) are present in an equimolar ratio and arise from an intramolecular recombination at a specific site within the inverted repeats [14,38,96,106]. This recombination is mediated by the product of a gene, called *FLP*, on the ABLE region [7,38,96,106]. This was demonstrated by the fact that a chimaeric plasmid containing 2μm DNA having the L and S sequences in one orientation was converted into the isomeric forms

in both orientations in yeast transformations [38,96,106]. The *FLP* function is *trans*-acting. Therefore on transforming a chimaeric *FLP*⁻ plasmid, the intramolecular recombination can occur in a strain harbouring a 2μm plasmid ([*Cir*⁺] strain), but not in a strain lacking the plasmid ([*Cir*⁰] strain) [14,38,96,106].

Early workers [13,14,28,38,91,96] suggested the cytoplasmic location for the 2μm plasmid on the basis of its absence in isolated nuclei, no homology to the genome by hybridisation, and its non-Mendelian inheritance. Yeast chromosomal genes carried on 2μm DNA chimaeric plasmids behaved as cytoplasmic elements at meiosis, but recent biochemical and genetic data strongly support the localisation within the nucleus.

One of the best methods of yeast transformation is based on the incorporation of exogeneous DNA into yeast protoplasts in the presence of polyethylene glycol (PEG) and the regeneration of protoplasts into 'normal' cells in hypertonic selective media. The transforming DNAs are generally grouped into two classes; (i) integrating and (ii) autonomously replicating types.

(i) Integrating plasmids are constructed by insertion of yeast genomic DNA (usually chromosomal DNA fragments containing a selective marker gene, such as *LEU 2*, *HIS 3*, and *URA 3*) into an *E. coli* plasmid. Plasmids of this kind, incapable of replicating autonomously in yeast cells due to the lack of a yeast DNA replicator, transform suitable nutritional mutants of yeast *S. cerevisiae*

with a low frequency of less than ten transformants per μg of DNA through chromosomal integration. Integration occurs in the homologous chromosmal region mostly by recombination [7,17,38,74,91]. This leads to uptake of the entire plasmid into the genome, resulting in the duplication of homologous yeast DNA sequences. For instance, an integrating plasmid YIp1 (a chimaeric plasmid between a bacterial plasmid pBR322 and the yeast *HIS 3* DNA fragment) integrates in the vicinity of chromosomal *his 3* locus with the creaton of duplicated *his 3* and *HIS 3* sequences [72,74]. In contrast, another integrating plasmid, pYeleu 10 (a chimaeric plasmid between ColE1 and the yeast *LEU 2* DNA fragment), integrated not only in the homologous *leu 2* locus, but also in several unlinked chromosomal sites [7,38,45]. This unusual integration event was interpreted as resulting from the presence of a yeast moderately repetitive sequence, Ty1-17, in the vicinity of the *LEU 2* DNA carried on pYeleu 10 [7,38,45,72]. Ty1-17 is structurally related to a yeast transposable element Ty1 flanked by δ sequences, and has six copies dispersed throughout the haploid genome [28,38,40,72,74,76]. Integration of pYeleu at the *leu 2* locus can occur also by a double crossing-over event, although the result is indistinguishable from reversion at the *leu 2* locus [38,72].

A plasmid sequence integrated by the Campbell recombination is somewhat unstable, because one copy of the duplicated yeast homologous sequences, either of chromosomal or plasmid origin, is prone to be looped out of the genome together with the integrated *E. coli* sequence at a fequency of approximately 1% per cell division by a reversal of the recombination process by which the plasmid is integrated [38,72,91]. This insertion and excision principle led to

the exploitation of a method by which a wild-type yeast genome DNA can be replaced efficiently with DNA modified *in vitro* by deletion, insertion, or substitution [38,72].

Transformation fequency of integrating plasmids was enchanced in two ways. First, it could be achieved by linearisation of plasmids with restriction enzymes [53]. When plasmids were digested with enzymes that gave single cuts within the yeast DNA regions, the resulting linear plasmids showed transformation of five to twenty fold over that obtained with uncut circular plasmids. The effect may be ascribed to the fact that the broken ends of DNA molecules are highly recombinogenic and are prone to interact with the homologous genomic sequences by strand inversion and repair DNA synthesis during the recombination [38,53]. Secondly, a higher transformation frequency was obtained by inserting a segment of rDNA sequence into integrating plasmids [38,53,72,96,97]. The *S. cerevisiae* rRNA genes contain about 140 copies of tandemly repeated 9-kb units, mapped on chromosme XII [28,74] and, on this account can afford to provide multiple integration sites for plasmids carrying the homologous rDNA fragment. Transformation frequency was increased 100 to 200 times [28,38,106].

(ii) Integrating plasmids are converted to autonomously replicating plasmids, capable of efficient transformation, by inserting yeast replicator sequences from 2μm plasmid, chromosomal and mitochondrial DNAs.

Chimaeric 2μm-DNA containing plasmids are constructed by joining *E. coli* plasmids (usually pBR322 derivatives) with the entire sequence or a portion of 2μm and a selective yeast marker gene [7,14,38,72,91,96,97]. The chimaeric plasmids autonomously replicate in yeast cells in circular form and can be used as 'shuttle vectors' between yeast and *E. coli*. Yeast transformants, obtainable with a high frequency of 10^3-10^4colonies per μg of DNA, are unstable and 10-30% of the transformants lose the plasmid after 15-20 generations under non-selective conditions [7,10,14,96,105]. One likely reason for the plasmid instability is its replicative competition with the endogeneous 2μm plasmid, compared with more stable maintenance seen in Cir^0 strains [38,72,91,96]. Chimaeric plasmids carrying 2μm-DNA display non-mendelian segregation at meiosis, but probably exist within the nucleus and undergo homologous recombination with the resident 2μm-DNA or with chromosomal DNA [38,72,96,97].

High-frequency transformation can be accomplished also with bacterial plasmids containing specific yeast genomic DNA segments [38,40]. One well-studied examples is YRp7, consisting of pBR322 and a 1.4 kb yeast fragment involing the *TRP 1* gene from chromosome IV [38,40]. The chimaeric plasmid transforms yeast *trp 1* mutant to *TRP 1* phenotype with a frequency as high as 10^3-10^4 colonies per μg of DNA and replicates autonomously as circular DNA, like 2μm DNA chimaeric plasmids, indicating that the *TRP 1* DNA segment contains its own replicator, ie the putative *ars* elements (autonomously replicating sequence) [38,40,72,91,105]. A number of yeast genomic DNA segments containing *ars* were isolated on the criterion of high transforming ability and mitotic instability of chimaeric plasmid (*ars* elements could therefore be

artifactual). Among them are *ars 1* around *TRP 1* gene *ars 2* around *ARG 4* gene, *ars 3* around *SUP II* gene , and *ars 4* around the centromere of chromosome IV [38,40,72,91,96,97,98,106]. Non-transcribed spacer regions of repetitive rRNA genes, the vicinity of glutamate tRNA gene, and some other unidentified genomic and non-genomic regions also contain *ars* sequences, ie mitochondrial *ars* containing plasmids[38].

Autonomously replicating chimaeric plasmids were mitotically and meiotically stabilised by inserting yeast centromere sequences [10,38,72,91]. The centromere is a highly specialised chromosome region that provides the site for attachment of microtubules and regulates the proper chromosome movement during mitosis and meiosis. Clark and co-workers [28,38] first isolated the yeast centromere sequence (*CEN 3*) from chromosome III using the overlap hybridisation technique [10,28,38,91] and constructed chimaeric plasmids containing DNA replicator (*ars 1* or *ars 2*) and the *CEN 3* sequence [10,38,72,96,97]. The chimaeric plasmids transformed yeast with high frequency and were stably maintained at mitosis under non-selectine condition. At meiosis, the plasmid predominantly segregated in a Mendelian fashion (2+ : 2-) without showing linkage to any normal chromosomes. Thus, the *ars-CEN* plasmids behave in yeast as stable mini-chromosomes in a controlled small copy number (usually one).

TRP RI circle, a stable minichromosome of another type, was constructed without a centromere sequence [10,28,38,72,74,91,106]. This plasmid was built up by intramolecular circularisation of a 1.4 kb yeast DNA segment excised from YRp7 with EcoRI digestion. Therefore consisting only of yeast DNA bearing *TRP 1*

and *ars 1* [28,72,74,106]. The centromere region is not included, but TRP1 RI circle is stably maintained in yeast 100-200 copies per cells in the absence of selective pressure and segregates in a non-Mendelian fashion at meiosis. The plasmid is organised in a typical nucleosomal structure and is probably compartmentalised within the nucleus [10,28,38,72,74,106].

So far, the studies on yeast plasmids have centered around 2μm DNA molecules and artificially constructed chimaeric plasmids of *S. cerevisiae*, mainly because most yeast species do not harbour their own plasmids. Increasing interest in yeast plasmids from the standpoint of molecular genetics and importance as DNA cloning vector elements have stimulated work on isolation and characterisation of new yeast plasmids. Such as linear DNA Killer plasmids (pGK1 plasmids), 3μm-DNA plasmids and 2μm DNA-like plasmids.

1.4 Yeast transformation.

Yeast transformation systems with suitable vectors provide a useful method for studying function of not only yeast own genes, but also of diverse heterologous genes. For instance, integrating plasmids such as YIp5 are handy tools to isolate functional *ars* elements from a variety of yeasts and non-yeast sources. With autonomously replicating vectors, various genes are introduced into *S. cerevisiae*, directly or after in vitro modification, to investigate the gene expression in this eukaryotic microbe. The scope of genetic manipulation with new host and vector systems is also expanding.

Although numerous fruitful results have been presented in *S. cerevisiae* transformation systems, it is considerably important to develop new yeast host-vector systems to broaden knowledge of molecular biology of yeasts and for wider application of gene manipulation. A highly improved exression of cloned heterologous genes may be anticipated through the use of the new yeast host cells of phylogenetically and genetically distinct backgrounds.

New yeast transformation systems have been developed in *Schizosaccharomyces pombe* [5,6,88] and *Kluyveromyces lactis* [94]. In an attempt to construct suitable plasmid vectors in these yeasts, it was found that the *S. cerevisiae* 2μm DNA transforms *S. pombe* with high frequency [5,6,88] and *K. lactis* only inefficiently [94]. *S. cerevisiae* genomic *ars* elements did not serve as replicators in these yeasts, showing there is some difference in specificity of DNA replication mechanism among yeasts. Because of this, some efficent *ars* segments were independently looked for and isolated in these yeasts. Extrachromosomal 3μm DNA from *S. pombe* could be utilised as a vector in *S. pombe* [38,72]. The linear pGK1 plasmids are an interesting candidate for *K. lactis* vectors.

1.5 Promoters.

1.5.1 Eukaryotic gene promoters.

Characterisation of the nucleotide signals that constitute eukaryote promoters is essential to the understanding of gene regulation. Although we have a good understanding of the DNA sequence elements which constitute prokaryotic promoters, their eukaryotic counterparts are poorly defined .

The eukaryotic promoter for RNA polymerase II appears to have a complex structure. Now at least four distinct regions have been delineated [12,46,47]. The start site (cap site) identifies the point at which RNA transcription commences. The TATA site is a short AT- rich region about 30 bp upstream from the cap site, and its main function appears to be to accurately position initiation of transcription. The -50 to -90 region often contains the "-80 homology sequence" ie the CAAT box, and is required for efficient initiation, conferring the level of transcription (RNA polymerase attaches here). The RNA polymerase can cover a length of DNA over 50 nucleotides long, the possibility exists, therefore, that the polymerase has a "footprint" over the CAAT and TATA boxes. Plants have an additional complication, they may contain an "AGGA" box in plant promoter sequences.

Unexpectedly, indispensable expression sequences yet further upstream have been identified. For instance, results obtained with a number of deletion mutants have indicated that the most important element for SV40 early gene

expression lies within a 72 bp repeat region that starts about 115 bp upstream of the mRNA start sites [12]. Strikingly, this promoter element can potentiate (activate, enchance) expression from heterospecific promoters, even after a separation of thousands of base pairs has been introduced [12]. These elements, termed enhancers, are distinguished by their ability to activate transcription units independently of their orientation or precise positioning relative to the unit [12]. Enhancers operate in a variety of cell types, although not necessarily with equal efficiency, the polyoma enhancer for example, is functional in differentiated mouse cells but not in undifferentiated embryonal cells [12].

Differentiated cells in eukaryotes possess a remarkable capacity for selective expression of specific genes. A single gene may account for a large fraction of the total gene expression in one cell type, yet be expressed at undetectable levels in other cells; such as that regime found in plant seed proteins. The variation in expression is very large, and may approach a million-fold [12]. Current evidence suggests that the rate of transcription is a key aspect in the control of gene expression [12]. It is probable that evolution has concentrated its efforts towards the promoter sequences, providing a vast potential for variation in control and regulation within those sequences.

1.5.2. Yeast promoters.

Due to the difficulties in obtaining heterologous gene expression in yeast a number of groups have turned to the use of yeast promoters and translation initiation signals. This seems commonsense, in view of the fact that there is

variability found in both intra- and inter-specific functioning in pro- and eukaryotes alike. Thus expression of the *E.coli* β-galactosidase gene was obtained by fusing it to the N-terminus of the *ura3*, *cyc1* and *arg3* genes [72,80,110], and in yeast by fusing the α interferon gene to either the *pgk* or *trp1* genes [24,60,72].

In many instances, expression of a mature protein rather than a fusion protein is required. To achieve this Hitzeman *et al.* (1981)[16,60,72] started with a plasmid carrying the promoter and part of the coding sequence of the *adh1* gene. Six different promoter sequences were isolated (containing various deleted *adh1* promoter sequences), joined to an α interferon gene and used to direct the synthesis of mature interferon in yeast. In a similar fashion synthesis of mature hepatitis B virus surface antigen was achieved from the *adh1* promoter [71,82], and mature γ interferon from the *pgk* promoter [27,60,71,72].

In all three instances where a mature foreign protein was synthesized in yeast the levels of expression were considerably less than that of yeast alcohol dehydrogenase 1 (*adh1*) or 3 phosphoglycerate kinase (*pgk*). There are a number of explanations for this phenomenon, but none truly account for the lowered levels of expression found in heterologous systems [16,27,59,60,72].

In an attempt to identiy those structural features which control expression of yeast genes, Dobson *et al.* (1982)[24] compared the nucleotide sequence of the 5' flanking regions of 17 yeast genes. One sequence thought to be important for transcription initiation in eukarotes was found in most of the

yeast genes. This is the TATA box which is an AT-rich region with the canonical sequence TATAT/AAT/A usually located 25-32 bp upstream from the transcription initiation site, and A is never found at the begining. Functionally it is equivalent to the Pribnow box of prokaryotes, however, other sequences in eukaryotes were missing. With regard to translation initiation all the yeast genes had an adenine residue at position -3 and all except one had a pyrimidine, usually thymine, at position +6. The poor expression of the mature hepatitis antigen and interferons α and γ could be explained by poor translation initiation since in each case one of these residues was not conserved.

One if the most fascinating control mechanisms operating in yeast is that of the mating type swtch, the interconversion of yeast cells from one mating type (a or α) to the other [11,33,50,55,72,76,77,90,106,109]. This of particular interest as it may provide an important model for a mechanism of cellular differentiation. The development of yeast transformation techniques has led to the cloning of a set of genes involved in mating type interconversion and the biochemical confirmation of the " cassette model " for mating type switching which had been proposed on the basis of complex genetic analyses [11,33,50,55,76,77,96,97,109]. In this model the genes controlling yeast cell mating type reside on transposable elements which are only expressed when transposed from silent storage sites to the mating type locus some distance away on the same chromosome. It has become apparent from DNA sequencing studies of the cloned mating type genes that a simple model control of transcription through closely linked promoter sites cannot explain the

differential regulation of transcription of identical genes occupying different locations on the genome, and position effect models of gene expression were investigated by Klar *et al.* (1981)[106] and Nasmyth *et al.* (1981) [65,106].

Ideally, cloned genes should be placed downstream from a controllable promoter; such as that achieved by Kramer *et al.* (1984) [72]. They constructed a suitable vector using the promoter / regulator region of the repressible acid phosphatase gene of yeast, with the interferon gene inserted .

It must be realised that control of gene expression in yeast is much more complicated than is apparent from the above discussion. In-vitro mutagenesis of cloned genes followed by their reintroduction into yeast has resulted in the identification of *cis*-acting elements that modulate transcription despite the fact that they are located hundreds of nucleotides upstream of the site of transcription initiation [50,72].

1.5.3 Upstream Activator Sequences (UAS)

Transcription of most eukaryotic structural genes depends on DNA sequences located >100 bp from the initiation site of RNA synthesis [44,98,109]. Such sesquences (termed upstream promoter elements, modulators, enhancers, potentiators, upstream activator sequences) are not found in prokaryotic organisms. Generally, these upstream regions are necessary but not sufficient for maximal levels of transcription; the highly conserved TATA element located 25-30 bp upstream from the initiation site is also required. Enchacer elements

can work in either orientation and when located downstream from the transcriptional initiation site. In some cases, these sequences are also regulatory sites; they activate transcription only under certain physiological conditions, such as in response to hormones, or only in specific cell types. It is supposed that enhancer sequences are the critical elements that regulate gene expression during normal and abnormal development of multicellular organisms.

In yeast, transcription depends on TATA box sequences as well as upstream regions that have no fixed positional relationship to the initiation site or to the TATA box [46,98]. Some of these upstream regions serve as regulatory sites, one example of an upstream promoter/regulatory site is the region between the divergently transcribed *gal 1* and *gal 10* genes.

1.6 Stages in the yeast secretory pathway.

There is much of evidence that suggests that eukaryotic cells use a common pathway for secretion [25,26]. A series of membrane bound structures mediate the transfer of 'exported' proteins from their site of synthesis on the rough endoplasmic reticulum to their site of discharge, whether it be at the plasma membrane or into the vacuole. The process of intracellular protein transport and secretion involves several complex organelles and a large number of gene products [67,68,80,95,96,97]. In *S. cerevisiae* four stages have been detected that can be blocked reversibly in secretory mutants [41,67,69,96,97]. At a restrictive growth temperature these mutants accumulate organelles and glycoprotein precursors, which are discharged to a succeeding stage in the secretory pathway

upon return to a permissive temperature. The availability of the *sec* mutants provides a unique opportunity to examine the mechanism of intercompartmental transport. In yeast, Stevens *et al.* (1982) [95], previously established a pathway of organelles and glycoprotein intermediates that accumulate in mutants that are temperature sensitive for secretion and cell surface growth [67,68,69,95]. The results obtained by Stevens *et al.* (1982) [95] suggested that vacuolar and secretory glycoproteins require the same cellular functions for transport from the endoplasmic reticulum and from the Golgi body. The Golgi body represents a branch point in the pathway: from this organelle, vacuolar proenzymes are transported to the vacuole for proteolytic processing and secretory proteins are packaged into vesicles.

Compartmental modification of glycoproteins oligosaccharide chains has also been documented in yeast [41]. Core glycosylated forms of secretory proteins, such as invertase, are assembled in the endoplasmic reticulum (ER) and are modified further by addition of outer-chain carbohydate at some point during transit through the Golgi body [25,41,80,95]. This compares well with the use of vicilin constructs in yeast. Vicilin polypeptides, like those of legumin, are synthesized on rough endoplasmic reticulum, and cotranslational removal of leader sequences has been clearly demonstrated [20,21]. Like other vicilins, pea vicilin is glycosylated. This occurs in the ER, not in the Golgi apparatus [21,34,56,58], and the polypeptides of Mr 50,000 and Mr 16,000 are the main glycosylated components of mature vicilin.

1.6.1 The synthesis of Pea storage proteins.

Generally, storage protein is inert and often insoluble in aqueous media to allow deposition. It is syntheized specifically in the developing seed and is subsequently hydrolysed on seed germination to provide a source of amino acids and nitrogen to the growing seedling. The constraints on solubility and packing of storage proteins results in them often being large, multimeric molecules, whereas their role as a nutrient source is reflected in the high levels of nitrogen-rich amino acids, i.e., amides and arginine. Seed proteins can be divided into those that are necessary for the metabolism of the organism, and the accumulated storage material that has no apparent metabolic role. Although this distinction is not so clear cut. Storage proteins are often also distinguished from metabolic proteins on the basis of their solubility properties, since metabolic proteins are often water soluble (albumin type proteins), whereas storage proteins are usually only soluble in aqueous salt solutions (globulin type proteins), or in dissociating media (prolamin or glutelin type proteins). These distinctions can only be useful as crude descriptive nomenclature, since many exceptions are known.

Legumes in general contain globulin type storage proteins which can be assigned to two main types: Legumin and Vicilin. The general properties of these proteins have been reviewed previously [2,20,21,34,56,104]. Although of wide occurance, considerable variation in the relative amounts of these two types of storage protein exist between species, (e.g., *Phaseolus vulgaris* contains very little, if any, legumin, whereas *Vicia faba* contains a substantial excess of

legumin over vicilin [2,20,21,34,104]. The garden pea (*Fisum sativum*) is a typical legume about which there is a well-established body of knowledge including its genetics and physiology, and it is a crop of ecomonic importance: it contains significant amounts of both types of storage protein.

1.6.2. Storage proteins of pea.

Legumin and vicillin are divided on the basis of their solubility under different conditions of pH and ionic strength [21,34], this solubility fractionation does not yield pure proteins, a source of considerable confusion. Of the two storage protein solubility fractions in pea, the legumin fraction consists predominately of a single protein species -legumin- whereas the vicilin fraction as normally prepared contains two antigenically related protein species -vicilin and convicilin- both can be purified separately.

As the project deals with only vicilin constructs, these will be mentioed in more detail. Pea vicilin, Mr 150 to 190,000 [21,79]is dissociated by denaturants, e.g., SDS, into a large number of 'subunits', the major components being of Mr 50,000, 19,000, 16,000, 13,500, and 12,500 Mr by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). These polypeptides are present in the approximate molecular ratio 1 *(50,000)*: 1 *(33,000)*: 1 *(19,000)*: 2 *(16,000 + 13,500 + 12,500)* [21,34,56,79]. Minor components at 31,000 and 35,000 Mr are also usually present. (The subunits of convicilin of 71,000 Mr [21,34,56,79]). The molecular weights of vicilin subunits are not affected by denaturation in the presence of reducing agents, unlike legumin, and this is explained by the amino

acid composition of the protein which shows that it contains no measurable amounts of sulphur amino acids and thus can contain no disulphide-linked polypeptides. Vicilin, unlike legumin, contains small amounts of covalently bound carbohydrate [20,21,34,56,79].

The presence of a large number of different 'subunits' in vicilin made any derivation of a molecular structure for this protein difficult. Models that assume each of the observed polypeptides represented a separate entity and which proposed assembly of the molecules from six to seven separate 'subunits' [21,34] were sufficiently inpausible to suggest that the underlying structure was not as complex as the number of 'subunits' indicated. However, formulation of structural models for vicilin was dependent on study of its biosynthesis.

Vicilin shows heterogeneity at the molecular level, since it could be separated under non-dissociating conditions, e.g., chromatography on DEAE-cellulose or non-dissociating gel electrophoresis [21,34,56] into a number of molecular species containing slightly different subunit compositions, including a molecular species containing no 33,000 Mr subunit. It could also be separated into two fractions by carbohydrate affinity chromatography on immobilised concanavalin A, one fraction containing significant amounts of carbohydrate, and the other, not. The carbohydrate was shown to be associated predominantly with one subunit, that of 16,000 Mr by a variety of staining techniques after gel electrophoresis [34,56], and thus one fraction must contain vicilin molecular species with this subunit, whereas the other fraction contains the vicilin molecular species lacking it. Vicilin also shows heterogeneity in its component

polypeptides, and detailed analysis has shown the presence of charge variants of its subunits [21,34,56,79]. On two-dimensional isoelectric focusing: SDS-PAGE as many as 30 vicilin-derived polypeptides can be observed [212,56].

Antigenic similarity between vicilin and convicilin, but not legumin has been shown using antibodies raised against pea vicilin [21,34], this is not a clear indication of structural relatedness. Vicilin antisera will also react with homologous proteins in closely legume species, e.g., *Vicia*, *Lens*, etc. [34], but in more distantly related legumes, e.g., *Phaseolus*, immunoprecipitation of vicilin homologs cannot be demonstrated. Similar studies in the different pea vicilin molecular forms [34] have not shown any clear conclusions. Serological homology between the 16,000 and 12,500 Mr 'subunits' [21,34,56] has been shown, leading to the suggestion that these were glycosylated and non-glycosylated forms of the same or closely related polypeptide chains. This was supported by trypic peptide mapping studies on vicilin 'subunits' [21,34,56].

In vivo, storage proteins are synthesized within the storage parenchyma cells of the cotyledons on specially assembled rough endoplasmic reticulum, and are eventually deposited in membrane-bound storage organelles, called protein bodies [21,34,56,104]. The time from synthesis to deposition has been estimated to be approximately 1 hr [20,21]. The origin of these protein bodies is a matter of controversy, although clear evidence for the peripheral laying down of protein in large vacuoles at the early stages of protein synthesis has been presented [20,21,34,56,79]. A progressive change from 1 to 2 vacuoles, average diameter of 39 μm per cell to approximately 170,000 protein bodies, average

diameter of 1 to 2 μm per cell was observed as the seeds developed and it was suggested that the protein bodies had arisen as a result of fragmentation of the large vacuoles. Both legumin and vicilin were found in the same vacuoles or protein bodies [21,34,43,79,104]. However, probably not all protein bodies originate in this way as there is evidence in other legumes that dictyosome vesicles also appear to transport storage proteins to their sites of deposition [43,56]. A dual origin of the protein bodies in pea is thus possible, as has been suggested for *Vicia faba* [2,34,56,79].

Investigation of the molecular changes occurring in storage proteins during their synthesis, transport, and deposition in vivo has been carried out principally by pulse-chase labeling experiments on developing pea cotyledons with radioactive amino acids. The time scale of the process is sufficiently long to allow relatively easy observation of precursor-product relationship by following the movement of label into different polypeptides. The nature of storage protein precursor species was first indicated by translation of mRNA preparations isolated from developing pea seeds in cell-free translation systems in vitro.

1.7 AIMS and OBJECTIVES.

The elucidation of the regulatory mechanisms governing differential gene expression in eukaryotes is the major objective of modern molecular and developmental biology. The study of the control of gene expression in plants has been greatly facilitated by the advent of recombinant DNA technology, and

the availability of eukaryotic transformation systems. The large genome of most plants present difficulties in the isolation of specific genes, but molecular cloning has enabled the isolation of a number of these [73].

In order to extend the studies of the molecular biology of seed storage protein genes, and their expressional requirements; the expression of one of these genes, vicilin, was studied. The study took four forms:

(i) The expression of genomic DNA, with the associated splicing of introns, and the production of a functional protein in yeast.

(ii) The expression of cDNA using a variety of deletion vectors in yeast.

(iii) The use and comparison of transformation systems in yeast.

and (iv) Electronmicroscopic analysis of the resulting expressed proteins in yeast.

The strategies of attack on the various study forms can be seen in the results section, and the associated figures.

CHAPTER 2

MATERIALS and METHODS.

2. MATERIALS AND METHODS.

2.1 MATERIALS:

2.1.1 Chemicals and biological reagents.

All reagents, with the exception of those listed below, were from BDH chemicals Ltd., Dorest, U.K., and were of the analytical grade or the best available.

Acrylamide, bis-acrylamide, ampicillin (Ap), Bovine serum albumin (BSA), dithiothreitol (DTT), ethidium bromide (EtBr), herring sperm DNA, lysozyme, D-glucose, phenylmethylsulfonylfluoride (PMSF), RNase A, Lithum (Li) and sodium acetate (NaAc) were from Sigma Chemical Co., Poole, Dorest, U.K.

Sephadex G-50, Ficoll 400, and Percoll were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Caesium chloride (CsCl), and sodium chloride (NaCl) were from Koch-Light Ltd., Haverhill, Suffolk, U.K.

Nitrocellulose filters (BA85, 0.45 μm) were from Schleicher and Schull, Anderman and Co. Ltd., Kingston-upon-Thames, Surry, U.K.

3MM paper was from Whatman Ltd., Maidstone, Kent, U.K.

Bacto-peptone and Yeast nitrogen base with out amino acids were from Difco Laboratories, Detroit, Michigan, USA

Yeast extract was from Sterilin Ltd., Teddington, U.K.

Restriction endonucleases were from IBL, Northumberland, U.K., BethesdaResearch Laboratories (U.K.) Ltd., (BRL), Cambridge, U.K., The Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K., or New England Biolabs., CP. Laboratories Ltd., Bishops Stortford, Herts, U.K.

T4 DNA ligase, and Tris (Hydroxy methyl)aminomethane were from The Boehringer Corporation (London) Ltd.

Radiochemicals amd nick-translation kit N.5000 were from Amersham International p.l.c., Amersham, Bucks, U.K.

Plasmids and phage λ(NM258) used in the experiments described were supplied by Dr. M. D. Watson, from communal stocks.

All solutions, with the exception of electrophoresis buffers and solutions for Southern blotting, were sterilised by filtration or by autoclaving.

2.1.2 Bacterial and Yeast Strains.

Bacterial strains were derivations of *E. coli* K12. The yeast strains were of *S. cerevisiae* or *Schizosaccharomyces pombe*. Table (2.1) below lists these strains, plasmids and bacteriophage used as vectors or probes, and the sources or references for each.

Table 2.1 *E. coli* and Yeast strains, plasmids and Bacteriophage.

Bacterial Strain	Genetic character	Reference or Source
JM83	ara, Δ(lac-proAB) rpsL ø80, lacZ Δ M15	Dr. J. Messing.
JM101	Δlacpro supE thi F' traD36 proAB $lacI^q$ lacZ ΔM15	Dr. J. Messing.
Yeast Strains		
(i) *S.cerevisiae*		
MC16	αleu2-3, $his4\text{-}712^{FS}$, ade2-1, lys2-1, SUF2	DR.J. R. Fink.
MD40/4C	ura2, trp1, leu2-3,2-112, his3-11, 3-15	Dr. M. D. Watson.
MT302/1C	αleu2-3,112, ade1, arg1, ura1, pep4-3	Dr. M. D. Watson.

FS denotes a suppressible mutation, SUF2 is a suppressor gene.

(ii) *Schizo.pombe*

leu1-32h⁻	Dr. P. Nurse.

plasmids and vectors.

pUC8	Ap^R, lacZ	Drs. J. Vierra and J. Messing.
pMA257	See section 3.4	Dr. A. J. Kingsman.
pEMBLYe31	See section 3.1	Dr. G. Cesareni.
pJc2-7	See section 3.1	Dr. M. D. Watson.
pYSV9	See section 3.2	Dr. M. D. Watson.
pDUB2017	See section 3.4	Dr. M. D. Watson.
pDUB2018	See section 3.4	Dr. M. D. Watson.

Phages

λNM258 (C1857)	Dr. N. Murray.

2.1.3 Glass- and plastic-ware.

Glassware and plasticware used in manipulations of DNA, bacterial and yeast cultures, and for storage of sterile stock solutions and media, were autoclaved prior to use. Glassware, and plasticware used for very small amounts of DNA, or when very good recovery was required, was siliconised using 'Repelcote' (Hopkin and Williams, Romford, U.K.).

2.1.4 Growth Media.

The composition of the various media used for growth of the various organisms is given below (table 2.2):

TABLE 2.2 COMPOSITION OF GROWTH MEDIA.

MEDIUM	NUTRIENTS/litre
L broth	10g trypticase 5g yeast extract 10g NaCl
L agar	as L broth but 15g agar
YPD (broth)	10g yeast extract 20g D-glucose 20g bactopeptone
YPDA	as YPD but 15g agar
Yeast minimal media (YMM)	6.7g yeast nitrogen base w/o amino acids 20g D-glucose 20 µg/ml amino acid(s)
YMM agar	as YMM but 20g agar
YT broth	10g trypticase 5g yeast extract 5g NaCl
YT agar	as YT broth but 15g agar
YT/Amp/X-gal	YT ± agar containing 50µg/ml ampicillin and 40µg/ml X-gal

Regeneration agar (top layer/overlayer)	as YMM but 218g sorbitol (1.2M) 30g agar
Plate agar (PA)	as YMM but 218g sorbitol (1.2M) 20g agar

2.2 METHODS:

2.2.1 Biochemical techniques.

2.2.1.1 Phenol extraction of DNA samples.

Samples were deproteinised by addition of 1 vol. of redistilled phenol equilibrated with T.E. buffer (10mM Tris, 1mM ethylene-diamine-tetracetic acid (EDTA)) pH 8.0. Phases were mixed by vortexing for 30 sec. and separated by centrifugation at 12,000 *g* in a microfuge (MSE MicroCentaur) for 1 min., or, for larger samples, at 10,000 *g* in a Sorvall RC-5B centrifuge for 5 min. The aqueous phases were re-extracted with 1 vol. of phenol:chloroform:isoamylalchol (25:24:1 by vol). In some cases, the organic phases were re-extracted with 0.5 vol. of T.E. or T.E.S. (as T.E. but 10mM NaCl) buffer and the aqueous phases combimed. Phenol was removed either by 2-3 extractions with diethyl ether, or by 1 extraction with chloroform.

2.2.1.2 Precipitation of DNA with ethanol.

0.1 vol. of 3M sodium acetate (NaAc) pH 4.8 and 2.5 vol. of ethanol were added to the DNA solution and mixed. Samples were kept at -80°C for 30 min. or -20°C overnight. Precipitates were collected by centrifugation at 12,000 *g* for 5 min. in a microfuge, or at 12,000 *g* for 20 min. at 4°C in the Sorvall RC-5B centrifuge. Pellets were washed in cold 70% (v/v) etanol, dried briefly under vacuum and resuspended in a small volume of distilled water or T.E. buffer.

2.2.1.3 Dialysis of DNA solutions

Dialysis tubing (Medicell International Ltd., London, U.K.) was prepared by boiling for 10 min. in 2% (w/v) Sodium carbonate solution, rinsing extensively in distilled water, boiling for 5 min in distilled water and rinsing several times in distilled water. One end of the tubing was secured and the DNA solution pipetted into the tubing. The other end was secured, leaving a space to allow for an increase in volume. The tubing was placed in 2 litres of T.E. buffer and stirred at 4°C for 24h, with 2 further changes of buffer.

2.2.1.4 Preparation of denatured herring sperm DNA

500mg DNA was dissolved in 50 ml of distilled water by stirring for 2-4 h at room temperature. DNA was sheared by passing through a 19 gauge hypodermic

needle. The solution was boiled for 10 min.and stored at -20°C in small aliquots. The DNA was boiled for 5 min. prior to use.

2.2.1.5 Spectrophotometric quantitation of DNA solutions.

The optical density (O.D.) of DNA solutions were determined by adding 1µl of DNA solution to a quartz curvette containing 1ml of distilled water. 1µl of the buffer in which the DNA was dissolved was added to 1ml of water in a reference curvette, and the optical density from 320 to 230 nm recorded using a Pye Unicam SP8-150 uv/vis spectrophotometer.

The $O.D._{260}$ of a DNA solution of 1µg/ml is 0.02. A pure DNA sample has an $O.D._{260}/O.D._{280}$ ratio of ≈1.8 and the $O.D._{260}/O.D._{235}$ ratio is greater than this. The $O.D._{320}$ is zero. Deviation from these valves indicated the presence of contaminants such as phenol or proteins in the solution.

2.2.1.6 Storage of bacteria and yeast.

Both were stored for up to 6 weeks at 4°C on inverted agar plates tightly sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan). For longer storage, single colonies were streaked out onto selective agar plates and cells from the streak used to inoculate vials containing 1ml of L broth (Section 2.1.4). Cells were resuspended and 1ml 80% (v/v) glycerol added and mixed in. Vials were stored at -80°C. Bacteriophage were stored at 4°C in phage buffer (Section 2.2.2.1) saturated with chloroform.

2.2.1.7 Pretreatment of ribonuclease.

RNAase A was dissolved in water to a concentration of 10 mg/ml and boiled for 10 min. to inactivate DNAases. Small aliquots were stored at -80°C.

2.2.2 Enzymic reactions used in manipulations of DNA.

2.2.2.1 Restriction endonuclease digestions

The three buffer system shown below (table 2.3) was used [57,113].

TABLE 2.3. Restriction endonuclease buffers.

Buffer	NaCl	Tris/HCl pH7.4	$MgSO_4$	DTT
Low	0	10	10	1
Med	50	10	10	1
High	100	50	10	1

Core (10x) contains 0.54M NaCl, 0.54M Tris/HCl pH 8.0 and 0.11M $MgCl_2$.
Phage buffer as Med buffer.
Concentrations are mM, and stocks of 10x these were used.

DNA was digested with a 2-3 fold excess of enzyme. 0.1 vol. of a 10x stock of the appropriate buffer (Table 2.3) was added, and the volume made up with distilled water to 10x that of the enzyme. Plasmid and bacteriophage DNAs were incubated for 2-3 h at the appropriate temperature, genomic DNAs for at least 5 h. Reactions were terminated either by heating to 70°C for 5 min. or by addition agarose gel loading beads -stop dye- (20% (v/v) glycerol, 10mM

Tris/HCl, pH8.0, 10mM EDTA pH8.0, 0.2% (w/v) agarose, 0.1% (w/v) each of bromophenol blue, xylene, cyanol and orange G. The mixture was autoclaved and forced through a 19 gauge hypodermic needle to form beads).
(The buffer regime pertains to that found in reference 113).

2.2.2.2 Ligation of DNA fragments.

DNAs to be ligated were digested with appropriate restriction endonucleases, phenol extracted, ethanol precipitated and resuspended in a small volume of T.E. buffer pH8.0. Fragments were mixed to give a 3-fold molar excess of insert over vector. 0.1 vol. of 10x ligation buffer (0.66M Tris.HCl pH7.5, 50mM $MgCl_2$, 50mM DTT, 10mM ATP) and 2-5 units of T4 DNA ligase were added, mixed in and the tubes incubated at 15°C overnight. 0.1 to 0.5 vol. of the ligation mixes was used to transform competent *E.coli* cells, (Section 2.2.11).

2.2.2.3 Alkaline phosphatase treatment.

1 unit calf intestinal alkaline phosphatase was added to 13μg of either insert or vector DNA (Prepared as in section 2.2.2.4) in a total reaction volume of 40μl (recommended buffer: 50mM Tris-HCl pH9.5, 1mM spermidine and 0.1mM EDTA). This mixture was then incubated for 30 min. at 37°C. Nitrilotriacetic acid was then added to a final concentration of 10mM (This was added to chelate the divalent Zinc ions. The alkaline phosphatase is more thermolabile in the absence of Zinc ions). This was then heated for 10 min. at 70°C. The

mixture was then phenol extracted (Section 2.2.1.1) and ethanol precipitated (Section 2.2.1.2), and the pellet resuspended in 0.1 x T.E. at a concentration of 5μg/μl.

2.2.2.4 Preparation of the DNA.

Both insert and vector DNA were prepared by digesting them with the required restriction enzyme to give the required sites of attachment. After complete digestion EDTA was added to a final concentration of 15mM. The reaction was then heated for 10 min. at 70°C. DNA was recovered phenol extraction (Section 2.2.1.1) and ethanol precipitation (Section 2.2.1.2), washed and resuspended in T.E. at a final concentration of 0.5μg/μl.

2.2.3 Agarose gel electrophoresis

2.2.3.1 Large agarose gels.

Agarose was dissolved in 200ml of Alex's electrophoresis buffer (0.04M Tris-acetate, 0.001M EDTA pH7.7, 0.75μg/ml EtBr) by heating in a microwave oven for 4-5 min. The amount of agarose dissolved depended on the size of the fragments to be isolated (Table 2.4). The gel was then poured into a horizontal 180 x 150 mm perpex mould sealed to the glass plate with silicon grease. When set, the mould was removed and the gel placed in an electrophoresis tank containing the Alex's buffer. Samples were mixed with agarose gel beads

(Section 2.2.2.1) and located into the wells. Electrophoresis was overnight at 30V, or for 4-5h at 100-120V.

Table 2.4 Agarose gel concentrations.

Agarose gel concentration (%)	Efficient range of separation of linear DNA molecules (Kb).
0.3	60-5
0.6	20-1
0.7	10-0.8
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

2.2.3.2 Agarose minigels.

Agarose minigels were made using Tris/borate buffer (TBE, 0.089M Tris, 0.089M boric acid, 0.002M EDTA) and cast in a minigel electrophoresis apparatus (Cambridge Biotechnology Laboratories). Minigel were electrophoresed at 50V for 45-90 min.

2.2.3.3 Low melting point (LMP) gels.

The methology was as in section 2.2.3.1, except LMP agarose was used instead of ordinary agarose. Prior to loading the samples, the gel was pre-run for a period of 10 min., during this time the voltage was slowly increased to the required setting (this prevented splitting of the gel). The gel was run at up to 7.5V/cm.

2.2.3.4 Analysis of Band Patterns on gels to determine fragment size.

The sizes of dsDNA bands on agarose gels were estimated using various standard size marker; phage λ NM258 or C1857 cut with restriction enmdonuclease HindIII or PstI, plasmid pBR322 cut with HaeIII, pYSV9 cut with EcoRI and/or HindIII. The inverse of the logarithm (to the base 10) of the size of the fragment, in bp, was plotted against the distance of migration of the band in millimetres. This gave a slightly Sigmoidal-shaped curve which approximates very closely to three straight line segments, and allows accurate estimates of dsDNA fragment sizes from 100-5,000 bp.

Gels were visualised under short-wave ultraviolet illumination and photographed using a red filter and ASA 3000 film, Type 667 (Polaroid).

2.2.4 Transfer of DNA onto nitrocellulose or Nylon filters.

2.2.4.1 Southern blotting.

The method used was basically that of Southern (1975)[57,113]. The DNA in an agarose gel was made single stranded by shaking in denaturing buffer (1.5M sodium chloride, 0.5M sodium hydroxide) twice for 15 minutes. The gel was then neutralised by gentle shaking in neuralising buffer (1.5M sodium chloride, 0.5M Tris-HC1 pH7.4) twice for 15 minutes. A nitrocellulose filter was prepared by floating on distilled water until throughly wet, then soaking in 20 x SSC (3M sodium chloride, 0.3M sodium citrate adjusted to pH7.0 with sodium

hydroxide). At no time the filters handled except with rubber gloves and at the edges.

The nitrocellulose filter was laid on the gel, the position and orientation of the slots marked and a sheet of 3MM paper was wetted and laid on top, taking care to avoid "short circuit" liquid paths around the gel. Capillary action draws the 20 x SSC buffer up through the gel and filter into the paper nappies, transferring the DNA to the nitrocellulose where it sticks. The apparatus was always levelled and great care takem to exclude any air bubbles to avoid uneven transfer of DNA.

The process was complete after 12-24 hours. The DNA was fixed to the filter by washing in 6xSSC for 5 minutes, air drying in a sheet of 3MM paper and baking, sandwiched between 2 sheets of 3MM paper, at 80°C in a vacuum oven, for 2 hours. The filter was stored dry until used, protected between sheets of 3MM paper.

2.2.4.1 Prehybridisation of Nitrocellulose filters.

Filters were prewashed for 30 min at 42°C, and then prehybridised in the solutions indicated below for 1 to 4 h at 65°C. (Table 2.5)

2.2.4.1 Hybridisation to nitrocellulose filters.

The probe was denatured by boiling for 6-8 min., and was cooled rapidly on ice. The prehybridisation solution was removed from filters and replaced with a similar one containing 1.5 x Denharts' solution, before addition of probe. The probe was then added, and the filters hybridised for 4-24h at 65°C.

The filters were then post-washed in solution I (3 x for 10 min. at 42°C), and II (2 x for 30 min. at 65°C). The filters were then dried in a vacuum oven at 80°C for 1h. These filters were then autoradiographed (Section 2.2.7).

TABLE 2.5 Hybridisations solutions.

PRE-WASHING SOLUTION:	50mM Tris-HCl pH8.0 1M NaCl 1mM EDTA 0.1% SDS
PREHYBRIDISATION SOLUTION:	6 x SSC 0.5% SDS 5 x Denhart's solution 100µg/ml denatured Herring Sperm DNA ± 50% Formamide (deionised)
HYBRIDISATION SOLUTION:	6 x SSC 0.01M EDTA 5 x Denhart's solution 0.5% SDS 200µg/ml denatured Herring Sperm DNA ^{32}P-labelled denatured probe DNA ± 50% Formamide
POST WASHING SOLUTIONS: I	2 x SSC 0.1% SDS
II	1 x SSC 0.1% SDS

DENHART'S SOLUTION: 0.4% Ficoll
0.4% PVP
0.4% BSA

2.2.4.2 Western blotting.

The protien samples were primarily run on an SDS-PAGE gel, but were not stained (Section 2.2.15). The gel was placed in a try containing approximately 1 litre of Transfer buffer (0.45% w/v Tris, 2.16% w/v glycine, 30% v/v methanol), with a piece of nitrocellulose filter, for 1h.

A piece of ordinary 3MM paper was placed on the scrub pad of the blotting equipement, ensurring no bubbles were trapped. The gel was then placed onto the paper, with the nitrocellulose inbetween, forming a sandwich . This sandwich was sealed with elastic bands, and placed into the electrophoresis tank with 3 litres of Transfer buffer, and run at 60 V, 0.25 A for 4h.

The blot was then removed and incubated at 40°C for 1h with Horse Serum buffer (HSB- 2.5% w/v BSA, 0.05M sodium nitrite, and 5% inactivated horse serum; which was kept in the fridge, and heated to 30°C before use), in a tray with agitation, just covering the blot. The blot was then washed 3x in Tris-salt Buffer (0.5% w/v Tris-HCl pH 7.2, 1.8% w/v NaCl), incubated for 10 min. each time. The blot was then incubated for 1h at 40°C with 50µl 1° Antibody solution (Rabbit serum) in HSB, with agitation. This was again washed 3x in Tris-salt buffer, and then incubated for 1h at 40°C, with agitation, with 20µl

goat-anti rabbit in HSB (without sodium nitrate). The blot was then washed again. (Prior to the incubation with goat anti rabbit, 25mg DAB was added to 100ml Tris-salt buffer, heated at 65°C to dissolve the DAB, filtered, cobalt chloride and 10µl hydrogen peroxide added. This was mixed, added to the tray containing the blot, and agitated for approimately 2 min. or until the band(s) become visible.

2.2.5 ^{32}P-labelling of DNA by nick-translation.

The nick-translation kit (Section 2.1.1) was used according to the manufacture's instructions for labelling to a specific activity of 10^{8} c.p.m./µg, except that the volumes were adjusted so that 0.1 to 0.2µg of DNA was routinely labelled with 50 µCi of α[^{32}P]-dCTP in a volume of 25µl. Incubated at 15°C was for 2-2½h.

Labelled DNA was separated from unincorporated radionucleotide by passage through a 5-cm column of Sephadex G50 equilibrated in 150mM NaCl, 10mM EDTA, 50mM Tris/HCl pH7.5, and 0.1% SDS. 0.4ml fractions were collected and 1µl aliqupts counted in a β-scintillation counter. Fractions corresponding to the 1st peak of radio-activity contained labelled DNA, and were pooled for use as a hybridisation probe. Specific activities of > 1x10^{8} c.p.m./µg were routinely obtained.

2.2.6 Isolation of DNA fragments from agarose gels.

A number of methods were tried; the 3 given here were those most commonly used. For most of these methods, the DNA was digested with an appropriate restriction endonuclease, electrophoresed through an agarose gel, and required fragment cut out from the gel, trimming off excess agarose.

2.2.6.1 Low Melting Point (LMP) gel fragment isolation.

A LMP agarose gel was prepared, using the small gel surround, and placed in the gel electrophoresis tank, surounded by by Alex's buffer (Section 2.2.3.1).

To remove the required DNA fragment, the gel was first viewed under UV light, and removed from the gel with a scapel. The fragment bearing gel was then melted by heating it to 65°C, then 2-5 vol. of 50mM Tris-HCl, 0.5mM EDTA pH8.0 was added, mixed and placed at 37°C for a few min. An equal vol. of phenol was then added (phenol extraction -section 2.2.2.1), re-extracted using 24:1 Chloroform: isoamyl alcohol, and ethanol precipitated (section 2.2.2.2). Washed in 70% ethanol, dried under vacuum, and resuspended in sterile water, or T.E.

2.2.6.2 Freeze Elution fragment isolation.

The fragment was first cut out of the ordinary agarose gel, and put into a sterile Corex tube, 0.9ml water, 0.1ml 3M NaAc, 10mM EDTA added. This was left

in the dark for 15 min., with occasional gentle shaking. The eppendorf tubes were then prepared. (A small, 0.75ml, eppenorf was plugged with siliconised glass wool, and a holeput in the bottom and the lid. The eppendorf was then washed through with sterile water). The slice was then removed from the NaAc/EDTA solution, excess liquid blotted off, and the slice put into the small eppendorf(s), cutting the slice to size. These were then placed -80°C for 15 min. The small eppendorf tubes were then placed inside large (1.5ml) eppendorf tubes, and centrifuged in a microcentaur for 15 mins at 12,000 *g* (before the slices melted). 5µl of 1M $MgCl_2$, 10% acetic acid was added to the liquid. This was ethanol precipitated, twice, and resuspended in 20µl T.E.

2.2.6.3 Trough isolation of fragment

A slice was cut out of the gel in front of the required DNA fragment(s), and the trough filled with either T.E. or Alex's buffer. The gel was then put back into the electrophoresis apparatus, the Alex's buffer surrounding the gel was removed so that it did not cover the gel. The fragment was then electrophoresed until about half had entered the trough, the T.E. was then removed to an eppendorf tube, and kept. The trough was refilled, and the rest of the DNA run into the trough. The two fractions of T.E. were combined, phenol extracted, and ethanol precipitated, then resuspended in 50µl of T.E.

2.2.7 Autoradiography

Filters were taped to a piece of Whatman 3MM paper on a glass plate, the origin and sides marked with radioactive ink, and whole covered with cling film. Pre-flashed film (Fuji RX Satefy) and an intensifying screen (Dupont Lightening Plus) were placed over the filter (Laskey and Mills, 1977), followed by a glass plate. The assembly was secured with tape or elastic bands, wrapped in black polyethene bags and placed inside a light-tight box. Autoradiography was at -80°C for 30 min. to 3 weeks. Films were developed in Phenisol developer (Kodak) and fixed in Kodafix (Kodak).

2.2.8 Large scale preparation of plasmid DNA -BACTERIAL

10ml aliquots of either YT or L-broth, containing the appropriate antibiotic(s) (i.e. 50μg/ml ampicillin), were innoculated with cultures of the plasmid bearing strain(s) and grown overnight at 37°C on a rotary incubator. 250ml portions of the same growth media in 500-1000ml Sakaguchi flasks were innoculated with the overnight cultures (1:25 dilution) and grown with shaking at 37°C to an $O.D._{650}$ of 0.9. Chloroamphenicol was then added to a concentration 150-200μg/ml, to enhance the plasmid complement, and the incubation continued overnight.

The cells were pelleted by centrifugation at 15,000*g* for 10 min. at 4°C, and resuspended in 5ml of 25% w/v sucrose in 50 mM Tris-HCl pH8.0 at 0°C. Freshly prepared lysozyme solution (1ml of 10mg/ml lysozyme in the same

buffer) was added, mixed gently, and then incubated with shaking at 37°C for 2 min. and then at 0°C for 10 min. Next 5ml of 0.2M EDTA pH8.0 was added and shaking at 0°C a further 10 min., followed by the addition of 1ml of20% w/v SDS and gentle mixing at room temperature until the suspension clarified. NaCl (3ml of 5M in 50mM Tris-HCL pH8.0) was then added and the whole was mixed throughly and kept on ice for at least 4 h or usually overnight. Cell debris was removed by centrifugation at 38,000 *g* for 90 min. at 4°C, followed by careful removal of the supernatant with a pipette.

The plasmid DNA was precipitated from the supernatant by the addition of 50% w/v PEG 6000 in 50mM Tris-HCl pH8.0 to give final concentrations of 10% w/v PEG, 0.5M NaCl and 50mM Tris-HCl pH8.0. The mix was then stood at 0°C for at least 4 h or usually overnight, then gently centrifuged at about 2,000 *g* for 5-10 min. at 4°C. The pellet was drained throughly and resuspended in 4-5ml of T.E. buffer (10mM Tris-HCl pH7.5, 1mM EDTA) by gentle shaking at 0°C or if necessary by using a wide bore pipette. If this suspension was not clear it was briefly spun, it was kept at 0°C and could be stored.

The plasmid was purified by density gradient centrifugation; freshly prepared EtBr (10mg/ml in T.E. buffer) was added to a final concentration of 300μg/ml and caesium chloride to 48.4% w/v or a fractive index of 1.3890, the mixture was then stood in ice for 30-60 min. prior to centrifugation (Sorvall OTD65B Ultracentrifuge using a Beckmann VTi50 rotor) at 13,000 rpm for 30-60 min. at 0°C. The clear supernatant was then centrifuged in the same apparatus at 40-44,000 rpm for 20 h and the lower plasmid band removed with a wide bore

needle and syringe. This final step was repeated and the plasmid isolated by extracting with caesium chloride saturated isopropanol three times, dialysing against T.E. buffer (Section 2.2.1.3) and ethanol precipitation (Section 2.2.1.2).

2.2.9 Small-scale preparation of DNA.

2.2.9.1 Bacterial DNA minipreps (5ml).

This was essentially the method of Birnboim and Doly (1979)[57,113]. Single colonies were picked into 5ml of broth (L-broth for pBR322 type plasmids, YT broth for pUC8 plasmids) in M^{c}Cartney bottles containing the appropriate antibiotic(s) and were grown overnight at 37°C in a rotary incubator. The cells were spun down, in a bench centrifuge in the culture bottles, at 6,000 *g* for 15 min., then resuspended in 190µl of freshly prepared lysozyme solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8.0, 2mg/ml lysozyme), then a further addition 10µl of 50mg/ml lysozyme. This cocktail (200µl) was then transferred to a 1.5ml eppendorf tube and left at room temperature for 5 min., then chill on ice. Next 400µl of freshly prepared alkaline SDS solution II was added (0.2M NaCl, 1% SDS) and very gently mixed until the solution became sticky and viscous, the mix was then stood on ice for at least 5 min. NaAc solution (300µl of 3M pH8.0) was then added and gently mixed by inverting and then stood on ice for 5 min. The cell debris were then pelleted by centrifugation at 12,000 *g* for 5 min. (M.S.E. MicroCentaur bench centrifuge) and 750µl of the clear supernatant was transferred to a fresh eppendorf. Isopropanol (500µl) was added, gently mixed and the DNA precipitated at -20°C for 30 min. followed

by centrifugation at 12,000 *g* for 5 min. The pellet was washed in 70% ethanol, centrifuged and resuspended in 200µl of T.E.S., after removing all traces of ethanol. The solution was then phenol extracted (See section 2.2.2.1) and ethanol precipitated (See section 2.2.2.2), washed in 70% ethanol, vacuum dried and resuspended in either T.E. or HPLC grade water.

This method leaves large amounts of RNA in the DNA. 1µl of RNAase (0.1mg/ml)in a restriction digest allows down to 200bp to be detected on agarose gels by EtBr straining.

2.2.9.2 Bacterial DNA minipreps (50ml)

Single colonies were picked into 50ml L-broth cultures in the presence of the appropriate antibiotic, and grown overnight at 37°C. The cell were then spun down at 6,000 *g* for 10 min. in 50ml Sorvall tubes. The pellets were then resuspended in 2ml of solution I (See section 2.2.9.1), transferred into 30ml Corex tubes and left for 10 min. at room temperature. Then 4ml of solution II (See section 2.2.9.1), mixed gently by inversion, and left on ice for 10 min. Addition of 3ml ice cold 5M potassium acetate (KAc) pH4.8, and left on ice for 10 min. The mixture was then spun down at 6,000 *g* for 15 min. The supernatant removed into a fresh tube, phenol extracted twice, and ethanol precipitated. The pellet was then washed in 70% ethanol and vacuum dried. The 'clean' pellet was resuspended in up to 500µl of T.E., with the addition of DNAase free RNAase (50µg/ml) and removed to a 1.5 ml eppendorf tube.

10µl of this suspension could be restricted using 2µl core buffer (10x) (See section 2.2.2.1) and 1µl of the appropriate restriction enzyme, left at 37°C for 1-3 h. The remainder stored at -20°C.

2.2.9.3 Yeast DNA minipreps (5ml).

Single colonies were grown overnight in McCartney bottles containing 5ml YPD (See section 2.1.4). The cells were then spun down in a bench centrifuge, and resuspended in 0.5ml 1M sorbitol, 0.1ml 25mM EDTA, pH7.5; and transferred into 1.5ml eppendorf tubes. Then 20µl of either 2.5mg/ml Zymolyase 6,000 or 30µl 100mg/ml Novozyme was added, and incubated at 37°C for 60 min. After which the cells were spun down at 12,000 *g* in microcentaur for 1 min., the pellet was then resuspended in 500µl 50mM Tris-HCl pH7.4, 20mM EDTA, and 50µl 10% SDS, well mixed, and incubated at 65°C for30 min. KAc was then added (50µl 5M) and placed on ice for 60 min. The solution was then spun down at 12,000 *g* in a MicroCentaur for 15 min. The supernatant was then transferred to a fresh eppendorf tube and 1 vol. of isopropanol (at room temperature), gently mixed and left at room temperature for 5 min. The solution was then briefly spun in a MicroCentaur at 12,000 *g* for 10 sec. The supernatant was poured off and the pellet air dried. The pellet was resuspended in 300µl T.E. pH7.4. Addition of 15µl of a 1mg/ml solution of pancreatic RNase and incubate at 37°C for 30 min. was an optional extra. Then 30µl of 3M NaAc, mixed, and the DNA precipitated with 200µl isopropanol, and briefly spun to pellet the DNA. The supernatant was poured off and pellet vacuum dried and resuspended in 100-300µl of T.E.

Before using the DNA solution in a restriction digest, it may be necessary to spin the final solution hard (15 min.) in a microcentrifuge to remove insoluble material which may inhibit the restriction.

2.2.10 Subcloning into Bacteria.

2.2.10.1 Preparation of competant *E.coli* cells.

This method was obtained from Dr. C. O'Reilly. 10ml L-broth in a McCartney bottle was innoculated with *E.coli* and grown overnight at 37°C in a rotary incubator. This was used to innoculate a 100 ml L-broth in a Sakaguchi flask and the culture grown to an $O.D._{600}$ of 0.2 (about 1½-3h) at 37°C in a shaker. The culture was then spun down at 6,000 *g* for 10 min., washed in 40ml water, respun and resuspended in ½ vol. of 10mM NaCl, and respun. The pellet was resuspended in the same vol. of 30mM $CaCl_2$, respun and again resuspended in 30mM $CaCl_2$. This solution was then left for 20 min. on ice, and respun. The pellet was gently resuspended in 1/10 vol. 30mM $CaCl_2$, 15% glycerol. 200µl aliquots were taken and stored at -20°C.

2.2.11 Transformation of the competant *E.coli* cells.

DNA (to a maximum of 40 ng to give maximum efficiency) was added in ligation buffer (See section 2.2.2.2) or T.E. to each aliquot, and store on ice for 30 min. These were then heat shocked for 2 min. at 42°C or 5 min. at 37°C. Then 1ml of YT or L-broth (See section 2.1.4) was added and incubated at

37°C for an hour (This allows ampicillin resistance to develop). 0.1 vol. or 100µl of the solution was then spread onto appropriate selective plates, and left at 37°C for 24-48h.

2.2.12 Preparation of bacteria for colony hybridisations.

A modification of the method of Grunstein and Hogness (1975)[57,113] was used. Transformed colonies were picked off onto a grid drawn on a selective agar 'master' plate. Colonies were also streaked in the same positions onto a nitrocellulose filter placed on the surface of a fresh plate. The filter and plates were marked to aid orientation, and incubated at 37°C until streaks were visible. The 'master' plate was then placed at 4°C. (Another method involves blotting instead of picking the colonies on to the nitrocellulose, placing the filter onto a fresh plate and growing until visible).

The nitrocellulose filter was peeled off the plate and placed, colony side up, on filter paper soaked in denaturation solution (See section 2.2.4.1.2) and left for 5 min. The filter was further processed when placed on blotting paper soaked in neutralising solution, and then on blotting paper soaked in 2 x SSC, for 5 min. each time. The filter was baked at 80°C under vacuum for 1h, and hybridised to the ^{32}P labelled vicilin (See section 2.2.5). Positive colonies were picked from the 'master' plate and streaked onto fresh plates, or used to prepare plasmid DNA.

2.2.13 Yeast transformation.

2.2.13.1 Lithium acetate (LiAc) method.

Yeast cells were aerobically grown in 100ml of YPD (Section 2.1.4) within a 500ml Sakaguchi flask at 30°C (with shaking), usually overnight or until an $O.D._{610}$ of between 4 and 8 (corresponding to late log phase)(Section 3.3). Cells were harvested by centrifugation at 5,000 *g* for 5 min., washed once with T.E., and resuspended in the same buffer to the final concentration of $2x10^{8}$cells/ml. A 0.5ml portion of this cell suspension was transferred to a test tube (1.2 by 10.5cm), and an equal volume of 0.2M metal ions was added (LiAc). After 1h at 30°C with shaking, 0.1 ml of this cell suspension was transferred to a 1.5ml eppendorf tube and incubated with the addition of plasmid DNA solution (to a maximum of 670µg/ml) at 30°C for 30 min. An equal volume of 70% PEG-4000 (dissolved in water, sterilised, and mixed on a vortex) was then added, and left to stand for 1h at 30°C, after which the tube was immersed in water at 42°C and incubated for 5 min. The tube was immediately cooled to room temperature, washed twice in water and resuspended in 1.0ml of water. 100µl aliquots of this solution was then plated onto selective media, and the transformed cells left to grow at 30°C for 24-48h. The number of cells were then counted.

2.2.13.2 Via protoplasting/spheroplasting.

A 5 or 10ml culture of Yeast in YPD was grown overnight. This was then used to innoculate a 200ml culture, which then incubated for approximately 17h at 30°C with shaking. The cells were then washed in 100ml sterile water, and resuspended in 35ml SED (1.2M sobitol, 25mM EDTA, 50mM DTT, pH 8.0). The cells were pelleted (2,000 *g* for 10 min.), washed twice in 100ml 1.2M sorbitol, and resuspended in SCE (section 2.2.15.2). 0.3ml Glusulase, approximately 2.7 x 10 units, or Novozyme SP234, or 0.6ml Lyticase (5mg/ml, approximately 200 U/mg), and incubated at 30°C for 1h. The cells were now protoplasted and very fragile, and therefore were pelleted carefully, 1-2,000 *g* for 10-15 min. These protoplasts were washed 3x in 100ml 1.2M sorbitol to obtain an osmoticum, and resuspended firstly in 15ml SC (section 2.2.15.2), and then in 1ml. To 100µl of 'cells' up to 10µl DNA (2µg) was added. This was then left at room temperature for 15 min., when 1ml 20% PEG 4000, 10mM $CaCl_2$, 10mM Tris-HCl pH7.5. This was left no longer than 10 min. at room temperature. This was then pelleted at low speed for 20 sec., and resuspended in 100µl SC, 50µl YPD, 1.2M sorbitol, 40µg/ml leucine, and incubated for 20 min. at 30°C. This was then diluted (i.e. 10^{-3}) in 1.2M sorbitol and laid onto plate agar in 6ml regeneration agar. These were then incubated for 3-4 days at 30°C. Whence the numbers of transformed cells were recorded, and the plate kept for further analysis.

2.2.14 Electronmicrography.

2.2.14.1 Preparation of the yeast cells.

The preparation of the yeast cells was achieved by Miss Helen Grindley under the direction of Dr. N. Harris.

A two day old yeast culture on YMM agar was obtained, and the yeast cells were fixed *in situ* for 1h at room temperature in 3% paraformaldehyde, 1.25% glutaraldehyde, 0.1M phosphate buffer, and 0.1M sucrose. The cells were then left overnight in the same solution (except 1M sucrose was used). The next day a yeast colony was taken off the medium, using a razor blade, and transferred to a metal stub, which was immediately submerged in Freon Slush until completely frozen. Frozen sections were then cut on a Sorvall MT2-B ultra-microtome, at approximately -80°C, and picked up dry onto 200 mesh copper grids with carbon coated formvar film. These grids were floated on drops of 2.3M sucrose, 4% paraformaldehyde in standard phosphate buffer salts (PBS).

2.2.14.2 Immunogold labelling of prepared cells.

Grids were washed 3x on drops of 5% BSA in PBS, and incubated in Vicilin Antibody diluted 1 in 10, 1 in 20, and 1 in 50 in BSA/PBS for 30 min. (These were also washed 3x 5 min. on drops of BSA/PBS, with PBS washes inbetween). The sections were then incubated in either 5nm Protein A-Gold or 15nm Goat anti Rabbit IgG gold, diluted 1 in 200 with BSA/PBS for 30 min. Then washed 3x 5

min. on drops of BSA/PBS with PBS washes inbetween. These were further washed in water, and stained with aqueous Uranyl acetate (saturated solution) for 5 min., this was then washed with water.

Sections were then examined on a Philips 400 Transition Electron Microscope (TEM), and required photographs taken.

2.2.15 SDS-Polyacrylamide Gel Electrophoresis of Polypeptides.

A slab gel using spacers was made up, leaving the well-forming comb out at this stage. The longer spacer goes across the bottom, the two shorter ones up the sides. The spacers should be lightly greased to aid sealing. These are sandwiched with two glass plates, one with the cut-out at the top, and clamped together with bulldog clips. Two of which are put on the bottom, to enable the slab to stand up vertical without aid.

The separating gel acrylamide mix was prepared in the Buchner as follows; 22.5ml Tris-HCl pH8.8, 34.5ml separating gel acrylamide stock (30% w/v acrylamide, 13.5% w/v bisacrlamide). Degas the mixture (air inhibits polymerisation), and quickly add; 1.5ml ammonium persulphate (15mg/ml), 0.6ml 10% SDS, and 20µl TEMED (catalyst). Mix, pour the mixture into the gel slab immediately, up to a level approximately 2.5 cm below the cut-out. Now gently overlay with water to a depth of 2-3 mm, and leave to polymerise for at least 30 min.

The water was removed from above the separating gel, and make up the staking gel mixture, again in the Buchner flask, as follows; 2.5 ml Tris-HCl pH6.8, 2ml stacking gel arcylamide stock (30% acrylamide, 34.5% bisacrylamide), and 14.8ml distilled water. Degas and add 0.5ml ammonium persulphate (20mg/ml), 0.2ml 10% SDS and 20µl TEMED (Dye marker was also added-it was not added to the electrophoresis buffer). This was poured onto the separating gel to fill the slab, the well-forming comb gently put into the cut-out, and left to polymerise.

The comb was carefully removed, and the electrophoresis buffer prepared (Tris-glycine pH8.3, 0.1% SDS). The bottom spacer was removed and the gel slab clamped into the electrophoresis tank, 500ml of buffer was added to each reservoir, and the bubbles were removed from under the gel. The samples and standards were then added, with 1µl of the reducing agent 2-mercaptoethanol.

2.2.15.1 Preparation of protein samples.

Yeast cultures were grown overnight at 30°C in 10ml YMM, this was used to inoculate a Sakaguchi flask containing 200ml YMM, and incubated for approximately 17h at 30°C, with shaking. The cells were pelleted (5,000 *g* for 5 min.), washed in 100ml sterile water, and resuspended in 30ml SCE (1.2M Sorbitol, 10mM EDTA, 0.1M Sodium citrate, pH5.8). To this 0.3ml Glusulase (approximately 2.7 x 10 units), and incubated for 1h. The cells were then pelleted (2,000 *g* for 10 min.), washed 3x in 1.2M sorbitol, and resuspended in 15ml SC (1.2M sorbitol, 10mM $CaCl_2$), pelleted and resuspended in 1ml SC. Again

the mixture was pelleted and resuspended in Sample buffer (0.2M Tris-HCl, pH6.8, 2% SDS and 10% sucrose) at 2mg/ml, and boiled for 5 min.

10µl was loaded per gel slot.

2.2.15.2 Staining and Destaining.

The polyacrylamide gel was stained overnight in Stain (50% v/v methanol, 7% v/v acetic acid, and 0.075% w/v Kenacid R250), and destained with Destain (as stain but without Kenacid R250) overnight.

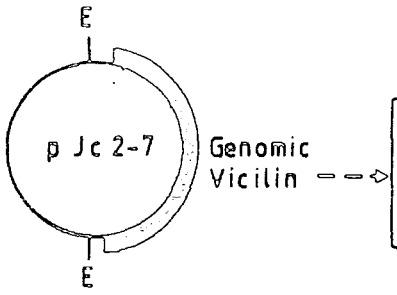
The gel was then vacuum dried, and the gel kept.

CHAPTER 3

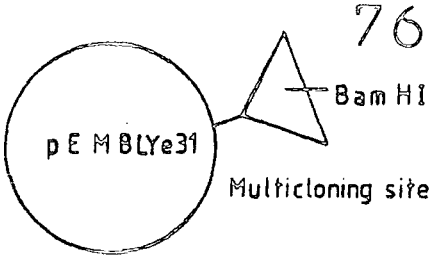
RESULTS and DISCUSSION.

FIGURE 3.1

The cloning strategy for the Genomic Vicilin recombinant plasmids.



Sequencing of pJc 2-7 plasmid DNA dideoxy method.



Linearisation with restriction enzyme (EcoRI)

Fragment isolation of genomic Vicilin

Modification of DNA termini

Filling up sticky ends with Klenow enzyme or digestion of sticky ends with Nuclease S1

Linearisation with restriction enzyme (Bam HI)

Modification of Vector termini

Dephosphorylation of Vector-alkaline phosphatase

Linker adaptation with T_4 DNA ligase - BamHI linker sites

Ligation of DNA and Vector with T_4 ligase

Recombinant DNA

Transform into JM83 and amplify under antibiotic selection. Host cell colonisation

Screening of host cells for cloned DNA eg. by colony hybridization

Restriction analysis - for correct orientation of genomic DNA in Vector

Transformation into Yeast

Southern blotting and hybridisation

pDUB 2017 or pDUB 2018

Restriction enzyme and fragment isolation

Vicilin c DNA

Generation of hybridization pr
- Nick translation DNA polyme
I

Transcriptional analysis

Expression of cloned DNA to produce protein

Western blotting and hybridization

Detection of protein eg: with test combination Gene expression

Electron micrograph studies using a Vicilin gold label.

FIG.3.1

3.1 Expression of genomic Vicilin sequences in yeast.

3.1.1 Aims and Strategy.

The aim of this part of the project was to study the expression of genomic plant genes in the yeast, *S. cerevisiae* and *S. pombe*. A comparison was to be made of the fidelity of expression, as measured by intron removal and subsequent translation of the resulting mRNA sequences into a fully functional protein. The overall strategy is shown in figure 3.1, and further elucidated and discussed below.

3.1.2 Production of the Recombinant Genomic Clone.

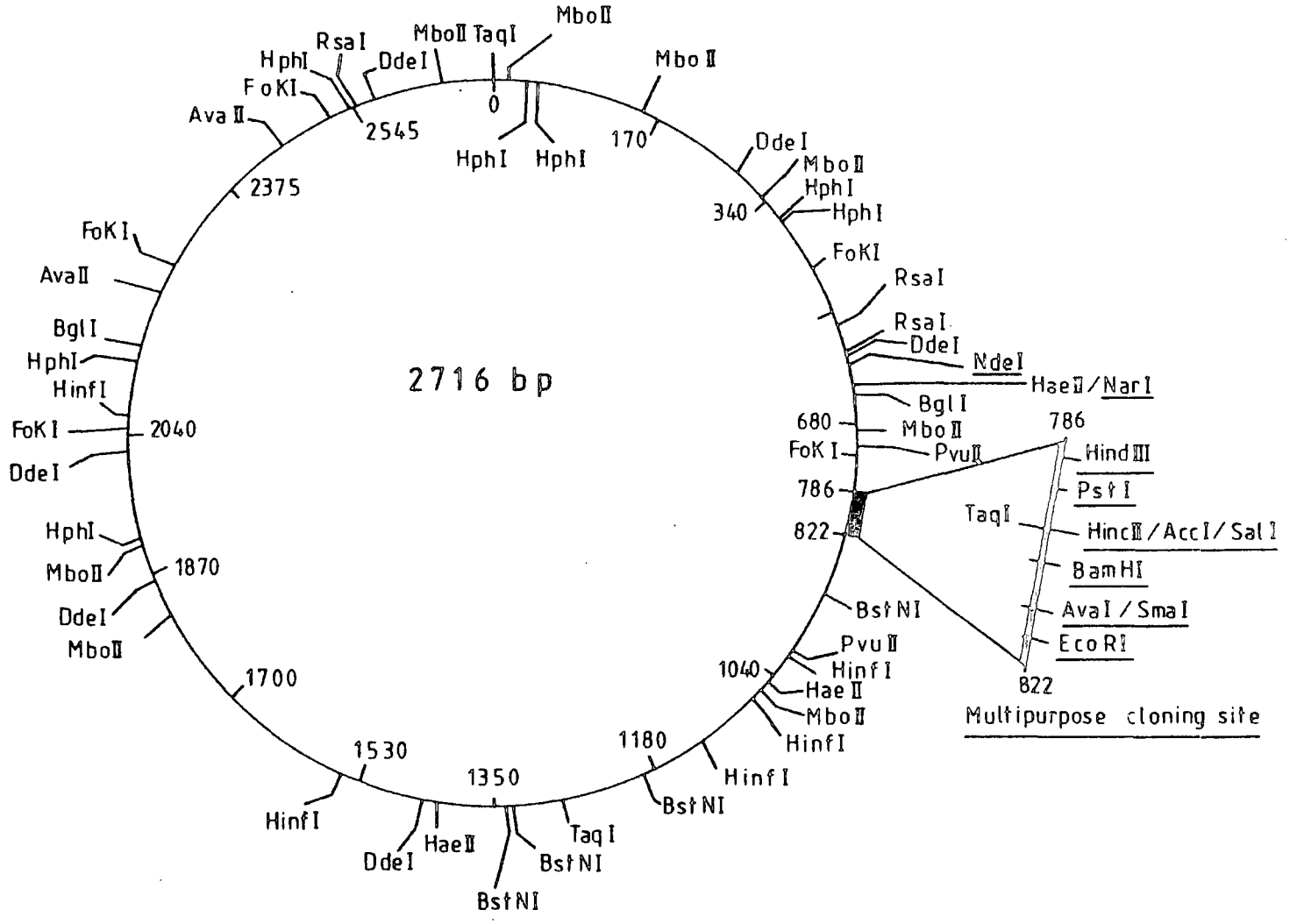
The plasmid pJC2-7 is a pUC8 (figure 3.1.1) derivative containing a 4120 bp EcoRI fragment of genomic DNA carrying a complete vicilin gene (figure 3.1.2). It was restricted with the restriction endonuclease EcoR1 according to the methology seen in section 2.2.2. The results of this restriction can be viewed in figure 3.1.3 A and B. These figures show that the fragments range from greater than that comparable to the vicilin fragment to smaller fragments than that of the pUC8 DNA cut or uncut. Prior to this, the vector plasmid, pEMBLYe31 (8098 bp), was prepared for the insertion of the genomic vicilin isolated from the pJC2-7 EcoR1 digestion above. It was planned to insert the vicilin into the multicloning site, see figure 3.1.4, at the BamH1 sequence as there is no suitable EcoRI site. Therefore, the vector was restricted with the restriction endonuclease BamH1 (section 2.2.2), after which the termini of the

FIGURE 3.1.1

Restriction map of plasmid pUC8.

FIG3.1

Restriction map of plasmid pUC8



200bp
StuI
EcoRI
HpaI
HpaI
BalI
StuI
PvuII
KpaI
SphII
BglII
StuI
EcoRI
BamHI
5' 2.2.1 cDNA Homology
5'
Putive coding region
3'

FIG3.12

FIGURE 3.1.2

The restriction map, homology, and putative coding region of the genomic vicilin DNA, surrounded by the plasmid pUC8. The whole plasmid contributing the plasmid pJC2-7.

FIGURE 3.1.3

Both A and B(i) show restriction digests of pJC2-7 with EcoRI, giving numerous bands. B(ii) shows plasmid pUC8.

FIG. 3.1.3

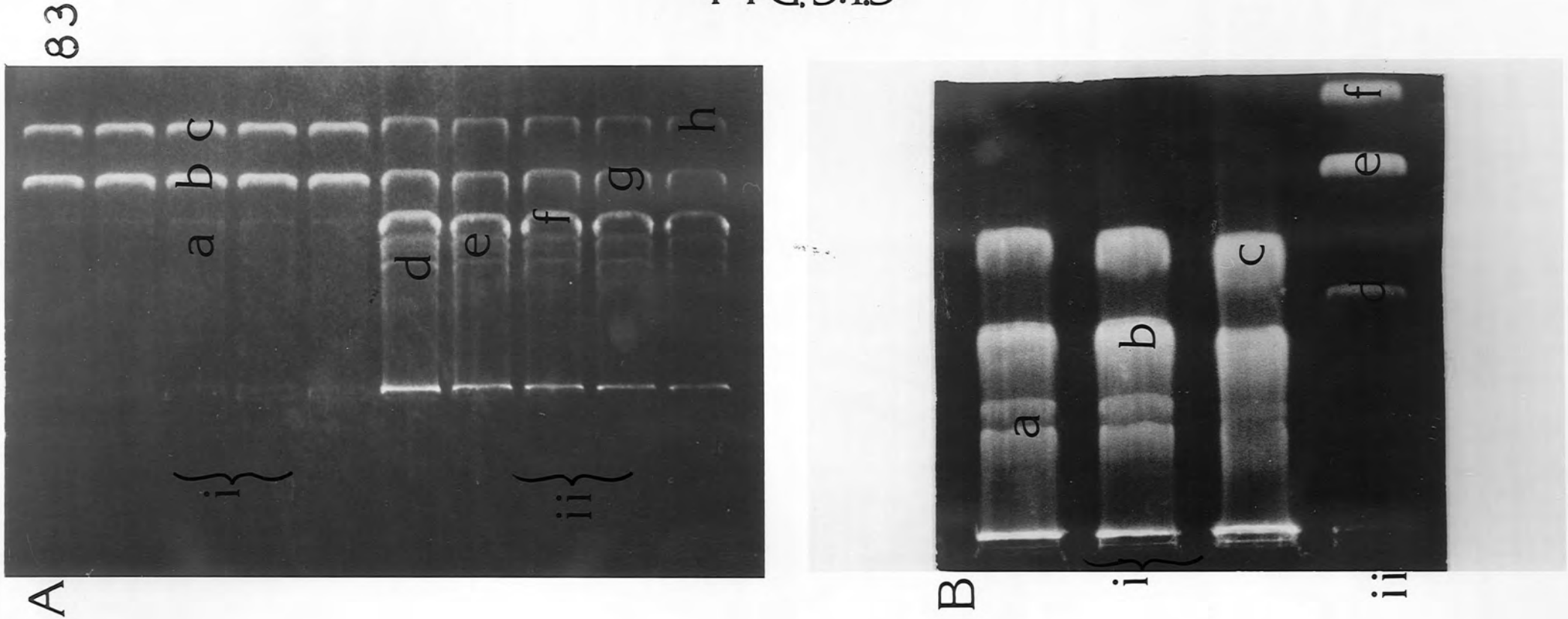
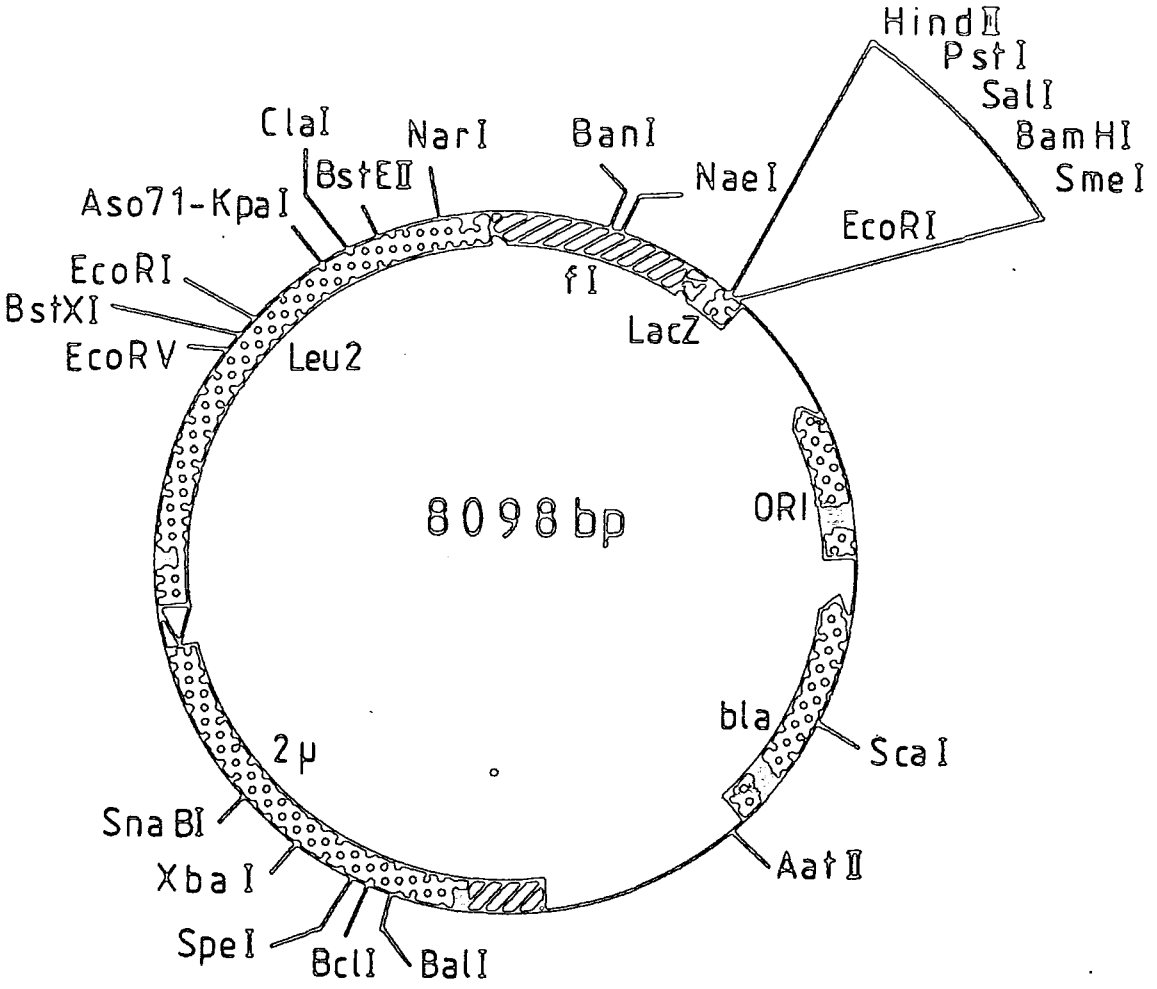


FIGURE 3.1.4

The restriction map of the plasmid pEMBLYe31.

FIG.314


HindⅢ
PstI
SalI
BamHI
SmeI
EcoRI
ClaI
NarI
BanI
NaeI
BstEⅡ
Aso71-KpaI
EcoRI
BstXI
EcoRV
Leu2
f1
LacZ
8098bp
ORI
bla
ScaI
2μ
SnaBI
XbaI
SpeI
BclI
BalI
AatⅡ

vector were modified, by phosphatase treatment (section 2.2.2.3). This part cloning production worked, but the restriction of the plasmid pJC2-7 did not go to plan. (See discussion). Therefore this part of the project was abandoned.

3.1.3 Discussion.

The restriction of the plasmid pJC2-7 proved to be difficult. The actual difficulty was seen when trying to isolate the vicilin genomic DNA. On restriction two bands should have been recorded, one (the largest Mr, and less mobile) corresponding to the genomic DNA, the other corresponding to the plasmid pUC8. These two bands were only seen once, figure 3.1.3 A(i), in the majority of cases numerous bands occurred (figure 3.1.3 A(ii) and B). This caused difficulty in the isolation of the correct band pertaining to the putative genomic vicilin. A variety of attempts to identify this fragment were made, i.e. running a standard along side the restriction; both cut and uncut pUC8, but this proved to be difficult as no fragment pertaining to this standard was seen. Figure 3.1.3 shows of these fragments, the variety observed, compared to those expected, could correspond to uncut, partially cut, and possibly fully cut pJC2-7 with EcoR1, on one hand; but may also correspond to the restriction of pJC2-7 at unknown site(s), or more likely EcoRI* (star) cleavage, therefore the isolation of the putative fragment of approximate size would not necessarily correspond to the required vicilin fragment. The star cleavage may be observed at low salt buffer concentrations (0-50mM NaCl)[113], but as advised medium to high buffer solutions were used.

Further DNA sequencing of this plasmid pJC2-7 would be required to ascertain the correct or full restriction map of this plasmid. The current map (figure 3.1.2), which was obtained from Mr. M. Lessaver shows the putative coding region, the 5' pRC2.2.1 cDNA region of homology (this corresponds to the cDNA coding for type A 50k vicilin obtained originally from pDUB2, whereas the cDNA described in Section 3.2 was cDNA coding for type C 50K vicilin obtained originally from pDUB9) [79,56,34]. This was not feasible as this would require a project in its own right.

Another reason to abandon this line of research was due to the difficulty observed when transforming the yeast *S. pombe*. (See section 3.3, the studies on transformation systems in yeast). Due these difficulties with *S. pombe*, it was decided that this yeast would not take any other part in the project, this decision was taken because the time of the project did not permit the development of the technique.

The idea for this type of study originated from a paper by Kaufer *et al.* (1985)[51], they studied heterologous gene expression in the fission yeast *S. pombe*. The yeast *S. cerevisiae* has been shown to be incapable of correctly removing intervening sequences from transcripts of higher eukaryotic genes [51,8,9]. This is probably due to the stringent requirement for the presence of a TACTAAC box close to the 3' end of the intervening sequence if splicing in *S. cerevisiae* is to occur [51,64,33]. There results demonstrated that *S. pombe* can correctly recognise the 5' and 3' splice sites of the SV40 small-T antigen pre-

mRNA. Table 3.1 shows the consensus of conserved sequences at intron-exon boundries.

Table 3.1 Conserved sequences at intron-exon boundries.

	5' splice site	Internal conserved sequence	3' splice site	ref.
Higher eukaryotes	G GTAAGT G	CTAAT G C	TAG	51,64,20.19.
S. pombe	G GTANGT	CTAAT G C	TAG A	51,33,87.
SV40 Large-T	G GTATTT	CTAAT	TAG	51.
SV40 small-T	G GTAAAT	CTAAT	TAG	51.

The correct 3' splice site was not recognised in the budding yeast *S. cerevisiae* and the 5' splice sites utilised only very inefficiently [51]. In *S. pombe* the 5' splice sites used to produce the large-T antigen mRNA was used very inefficiently, if at all (this was unexpected; since in lytically infected monkey cells this 5' splice site was used 2-3 times more frequently than the small-T antigen 5' splice site [51]). This was throught to be due to the differences in the consensus sequences - see table 3.1. None of the *S. pombe* introns sequenced to date has a thymine at nucleotide 5 at the 5' end. Alternatively, it is conceivable that although this organism can recognise higher eukaryotic splicing signals, it cannot cope with multiple 5' splice sites for a single 3' splice site. Thus, having identified the 3' splice site, perhaps through an interaction with the internal conserved sequence, the *S. pombe* cell may scan through the transcript until it identifies the first 5' splice site and

completes intron excision. A second splice site beyond this would then be used inefficiently, if at all.

The coding sequences of the majority of eukaryotic genes are interrupted by introns. The removal of intervening sequences by the process of RNA splicing is thus a fundamentally important step in gene expression, yet one which is only poorly understood at the molecular level. The most enigmatic aspect of this class of reaction is how the splice junctions are identified and juxtaposed, despite their separation in the primary structure by as many as 100,000 nucleotides (nt) [101].

Progress toward an understanding of this problem has been most rapid for the so-called group I introns which comprise the intervening sequences from nuclear and mitochondrial rRNA, many mitochondrial mRNAs, and several chloroplast tRNA precursors [100,101,62]. These introns share a set of highly conserved structural elements that dicate a specific folding pattern which results in direct apposition of the intron/exon borders by alignment against an 'internal guide sequence'. The 3D geometry of this structure plays an even greater role, in that several of these precursors have been shown to undergo autocatalytic splicing *in vitro* in the complete absence of protein [101,62,100]. Analysis of intron sequences in yeast have revealed three highly conserved elements required for splicing. These are the 5' conensus sequence GTAPyGT, the 3' junction sequence PyAG and the sequence TACTAAC which lies near the 3' junction and functions as a branch point [78,101,49,31,66,55]. Despite this stronger conservation at the level of primary sequence in yeast splicing

signals, the challenge of explaining accurate juxtaposition of splice junctions without the assistance of intron-mediated folding applies equally to lower and higher eukaryotes.

The missing pieces of the splice junction puzzle are likely to be found among the molecular components of the splicing machinery. Lerner and Steitz (1979) and Rogers and Wall (1980) [101,31] first suggested that 5' and 3' junctions could be aligned via complementarity between these sequences and a stretch of 18 nts. at the 5' end of the U1 snRNA. While the U1 snRNP binds to the 5' splice site [101,64], evidence does not support base pairing between the 5' end of U1 and the 3' splice site [101,31,37,52]. Rather, it appears that the 3' region of the intron provides binding sites for two snRNPs; the U2 snRNP binds and protects the branchpoint [101,37,52,55,66,31,49], U5 snRNP, probably the 3' splice site [101]. From these results, a reasonable conjecture is that the splice sites are positioned via the interactions of the snRNPs with one another. This is consistent with the observatio that splicing intermediates in yeast [101,52,66,31], as well as in HeLa cells [101,31,49,55,52,37], are associated with a rapidly sedimenting, presumably multicomponent, complex named the spliceosome.

Analysis of the RNA species generated during mammalian pre-mRNA splicing in cell-free systems has led to the formulation of a two-step model for intron removal [55,101,37,52,66,49,31]. Studies of yeast pre-mRNA splicing *in vivo* suggests that a broadly similar mechanism could operate in yeast [55]. (Splicing of yeast actin pre-mRNA *in vivo* leads to the formation of an intron lariat,

containing an RNA branch, and another lariat molecule analogous to the intron-exon 2 species described for the mammalian system.

Although plant genes have slightly different conserved regions compared to that of mammalian, it was throught that it would be interesting to see how the yeast could cope with a genomic plant gene, VICILIN.

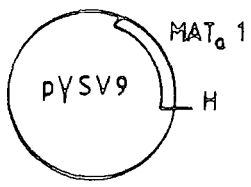
cDNA clones coding for vicilin have been produced on a variety of occasions [21,34,56,58], and on the basis of their sequences can be divided into three classes. One of them corresponding to the Mr 47K vicilin and the others to the Mr 50K vicilins, the DNA sequence homology between any two classes being about 85% [79,56]. The amino acid sequences determined from the DNA sequences confirm the model of synthesis of the vicilin subunits found *in vivo* and also explain why some vicilins are proteolytically cleaved and others are not.

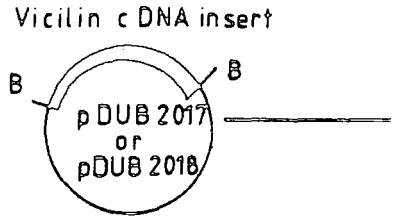
The 47K vicilin (vicilin B) contains a glycosylation site in its γ subunit and a proteolytic cleave signal at its β-γ junction, and hence is processed to the Mr 16,000 and 33,000 subunits. One of the Mr 50K vicilins (vicilin A) has no processing or glycosylation signals and hence is not processed. The other Mr 50K vicilin (vicilin C, this type is used in section 3.2) has no glycosylation site, but has two proteolytic cleavage signals at the α-β and β-γ subunit junctions, and hence gives rise to the Mr 12,500, 13,500, and 19,000 subunits [79,56,34,21]. This tolerably fits with the estimates of the molecular ratios of the subunits. Thus, the vicilin gene family consists of three types of vicilin gene, each of which is probably present in more than one copy: Delauney

(1984)[56] found minor DNA sequence differences in two otherwise identical vicilin C cDNAs. Domoney and Casey (1985) [34,56,73] estimated, by probing genomic DNA with cDNA probes, 5-7 vicilin B genes using their pCD4 Mr 47K vicilin cDNA clone and 4-6 genes for a Mr 50K vicilin using their pCD48 Mr 50K vicilin cDNA clone, which vicilin this codes for is as yet unknown. Significant cross-hybridisation of these two probe was detected.

It was hoped that the vicilin that was to be isolated from the plasmid pJC2-7, which corresponded to the type A 50K vicilin would have at least the same proteolytic and excision sites, producing the associated subunits. The pEMBLYe31 plasmid was obtained from pEMBLYe30 by deleting a non-essential PstI-SalI DNA fragment in the *LEU 2* region. The restriction map of the plasmid pEMBLYe31 is shown in figure 3.1.4. The plasmid pEMBLYe30 (9.5Kb) was constructed by insertion of the a yeast DNA fragment (5.5Kb XhoI-HindIII fragment of CV13), containing the *LEU 2* gene and the 2μ origin into the Cla I site of pEMBL9 [3,23]. It was planned that the vicilin would be inserted into the multicloning site at the BamH1 site, located in the *αLac Z* region of the plasmid. This would produce a recombinant plasmid of 12,218 bp, which would be selectable using Ampicilin and scoring insertional inactivation of the *αLac Z* gene (i.e. the formation of white colonies, instead of blue on Ampicilin X-gal plates). The pEMBLYe family of plasmids are therefore episomal, selectable, and can be transformed into yeast strains efficiently [3,23]. There are a variety of advantages that these plasmids offer;

(i) They are relatively small (5-10Kb) and, their complete nucleotide sequence can be reconstructed from the published data.





Linearisation with Restriction enzymes

Nibbling of ends with Nuclease Bal 31

Δ1 Δ7 Δ23 Δ26 Δ27

(Various deletions incurred)

Recirculation of Vectors

Transformation into JM83 amplification under antibiotic selection

Extraction of plasmid vectors and restriction digestion with Bam HI

Linearisation with restriction enzymes

Vicilin fragment isolation

B c DNA B

Generation of hybridization prol
- Nick translation ^{32}P labelled
DNA Polymerase I

Modification of vector termini
(i) Dephosphonylation of vector
Alakaline Phosphatase

Ligation of c DNA and vector with T_4 DNA ligase

Recombinant DNA
Δ1,7,23, 26,27

Amplification eg: under antibiotic selection Host cell colonization

Transformation of E Coli JM83 with Δ recombinant DNA

Southern blotting and hybridizat

Sequencing of cloned DNA dideoxy method

Screening of host cells for cloned DNA eg: by colony hybridization

Transformation into Yeast

Expression of cloned DNA into protein

Restriction analysis - correct orientation of vicilin c DNA into Vector

Detection of protein eg: with test combination gene expression

Transcriptional analysis

Western blotting

Electron micrograph studies using a Vicilin gold label

FIG.3.2

FIGURE 3.2

The strategy for the expression of vicilin cDNA in yeast.

(ii) They have a number of cloning sites conveniently located in the DNA sequence that encodes the α-fragment of β-galactosidase. Thus, *E. coli* clones harbouring recombinant plasmids can be easily identified.
(iii) All pEMBLY plasmids contain the intergenic region of the phage *f1*. Upon infection with *f1* their DNA can be encapsulated into phage rods and secreted into the medium in ss form [3]. This property is particularly useful for suh as sequencing experiments.

3.2 The cDNA clone.

3.2.1 Aims and Strategy.

The aim of this part of the project was to study the expression of plant cDNA genes in the yeast *S. cerevisiae* by measuring the fidelity of expression as judged by a functional complete protein. By doing this an insight into the possible use of *S. cerevisiae* for expressing heterologous plant proteins was to be made. The strategy for this part of the project can be viewed in figure 3.2

3.2.2 Production of the recombinant cDNA clones.

3.2.2.1 The creation of the deletions in Vector.

The plasmid pYSV9 (Figure 3.2.1) has the original size of 10,587 bp. It has a single BamHI expression site located at the C-terminal end of the prepro-sequence of the α mating factor. Upstream to this is a single HindIII site. The

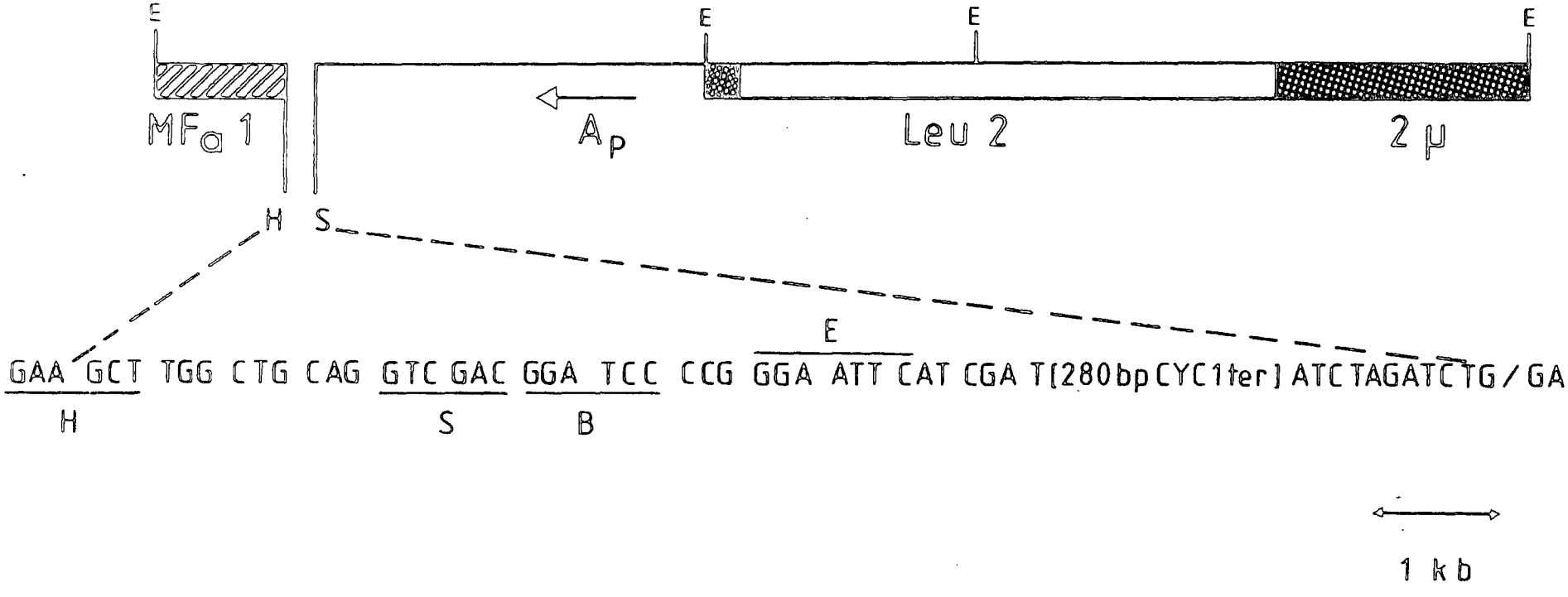

E
E
E
E
MFα 1
Ap
Leu 2
2 μ
H
S
E
GAA GCT TGG CTG CAG GTC GAC GGA TCC CCG GGA ATT CAT CGA T[280bp CYC1 ter] ATC TAGATC TG / GAC
H
S
B
1 kb

FIG 3.2.1

FIGURE 3.2.1

The restriction map of the plasmid pYSV9 showing;

-the α Mating factor 1 gene (MFα1)

-2μ plasmid

-Leu 2 gene

-Ampicilin gene (Ap)

-DNA sequences of the multicloning site between restriction sites for HindIII (H) and SalI (S)

E=EcoRI, B=BglII sites

FIGURE 3.2.3

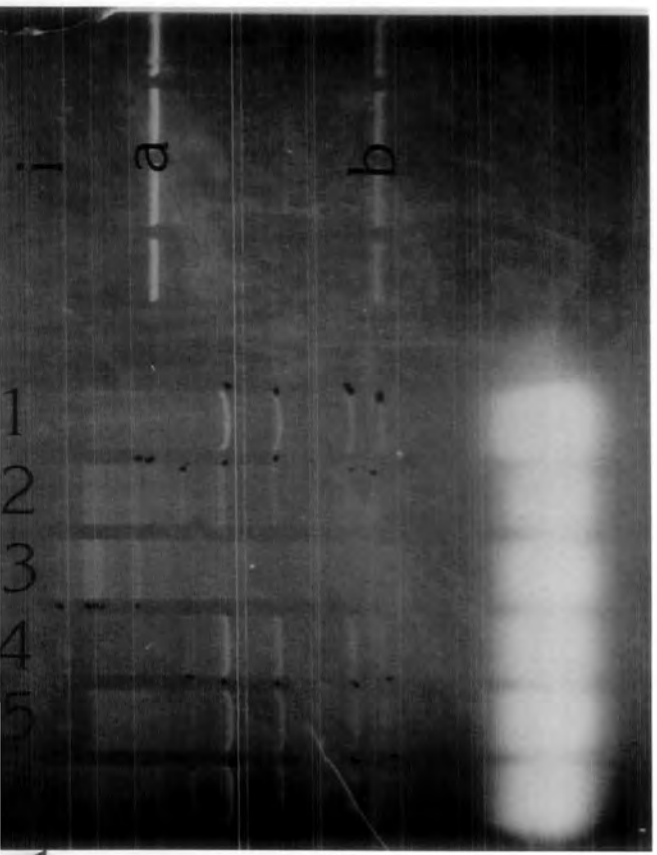
[A] (i) pDUB2018 BamHI restriction - two bands are produced a (cDNA vicilin, 1.43Kb) and b (pMA257)

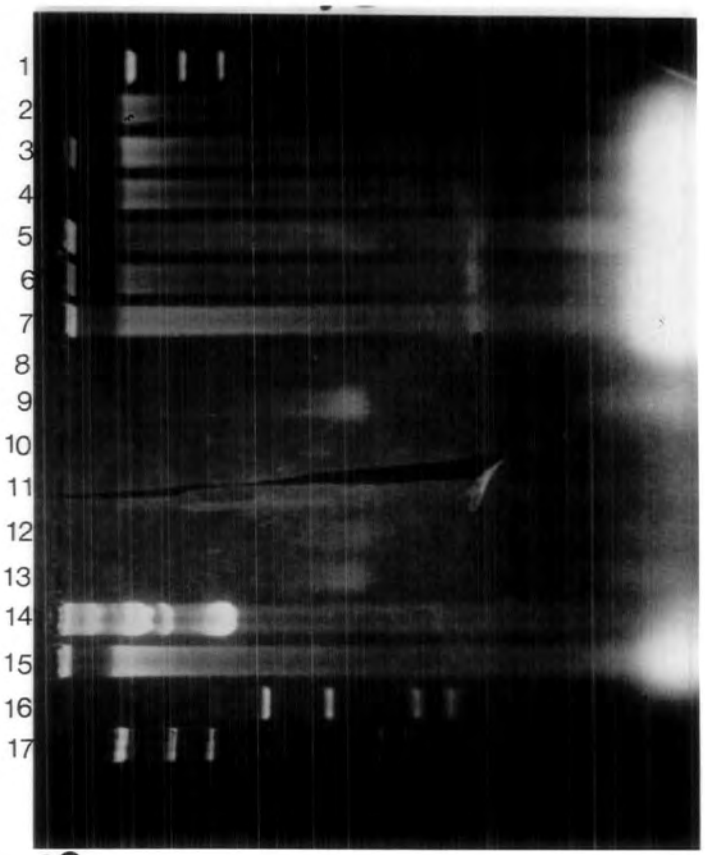
1 = pL1 E
2 = pL2 E
3 = pL3 E
4 = pL4 E
5 = pL5 E
6 = pL6 E (where E = EcoRI, B = BglII, H = HindIII)

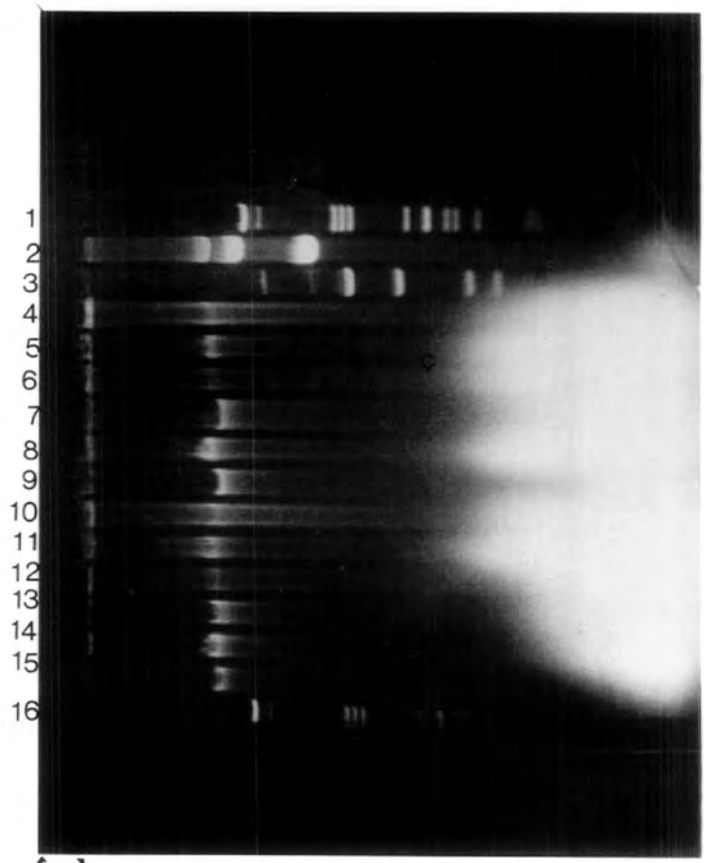
[B] 1 = 17 = λHindIII
2 = 8 = pL1 E/B
3 = 9 = pL2 E/B
4 = 10 = pL3 E/B
5 = 11 = pL4 E/B
6 = 12 = pL5 E/B
7 = 13 = pL6 E/B
14 = pYSV9 B
15 = pYSV9 E/B
16 = pYSV9 E

[C] 1 = 16 = λPstI
2 = pYSV9
3 = pYSV9 E
4 = 10 = pL6 E/H
5 = 11 = pL5 E/H
6 = 12 = pL4 E/H
7 = 13 = pL3 E/H
8 = 14 = pL2 E/H
9 = 15 = pL1 E/H

FIG.3.2.3


99
i
a
b
1
2
3
4
5
A


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
B


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
C

vector was first linearised with HindIII restriction endonuclease, and then treated with nuclease Bal31 for a few seconds. This degrades only a few bases and as a consequence removes the HindIII site. After religation and transformation, resultant clones were screened for sensitivity to BamHI and resistance to HindIII.

As a result of this treatment a series of deletion vectors were isolated; i.e. deletions (Δ) 1, 7, 23, 26, and 27. It was planned to sequence these vectors later, once the cDNA insert was present. The Δ vectors should now have a frame-shift, the product of this was to be seen later in the analysis of the protein expression.

3.2.2.2 The Production of the Recombinant DNA.

These vectors were separately re-introducted into *E. coli JM83* by transformation for amplification purposes, under antibiotic selection, extracted by use of plasmid miniprep techniques (Section 2.2.9), and the plasmids restricted with BamH1. The termini of these Δ vectors were then modified by dephosphorylation using Alkaline phosphatase treatment (Section 2.2.2.3). At the same time the plasmids pDUB2017 and pDUB2018 restricted with the endonuclease BamH1 to produce a cDNA vicilin fragment which could be introduced into the Δ vectors. On restriction the plasmids (pDUB2017 and 2018) gave two fragments (figure 3.2.3 A(i)), one corresponding to the vicilin fragment of 1.43Kb (b), and the other the plasmid pMA257 (a). The fragment was then isolated using one of the three methods shown in section 2.2.6, and used for two purposes;

FIGURES 3.2.4 to 3.2.6

These figures show colony hybridisation using the vicilin cDNA as a probe.

3.2.4 = the positive control

3.2.5 = putative colonies containing the vicilin

3.2.6 = as 3.2.5

FIGURES 3.2.4 to 3.2.6

These figures show colony hybridisation using the vicilin cDNA as a probe.

3.2.4 = the positive control

3.2.5 = putative colonies containing the vicilin

3.2.6 = as 3.2.5

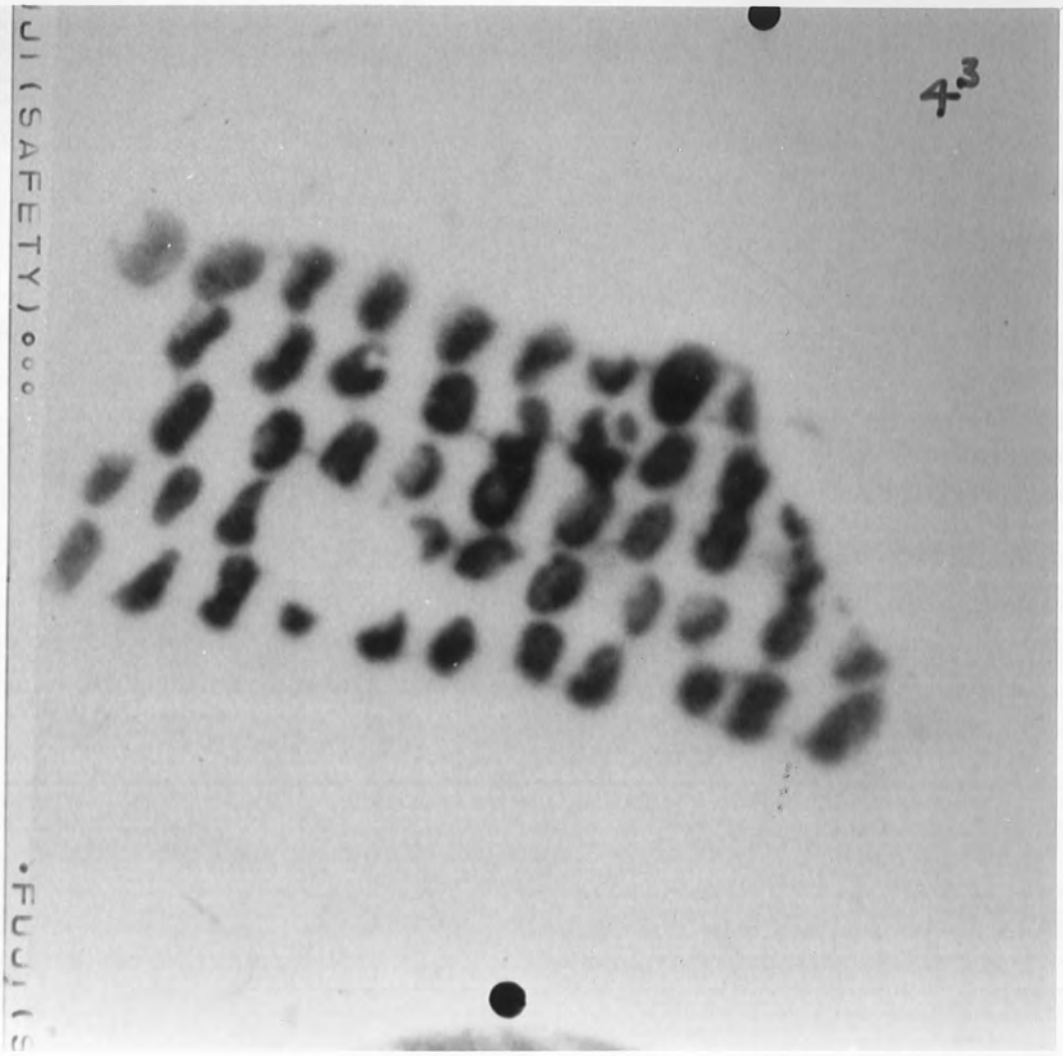


FIG.3.24

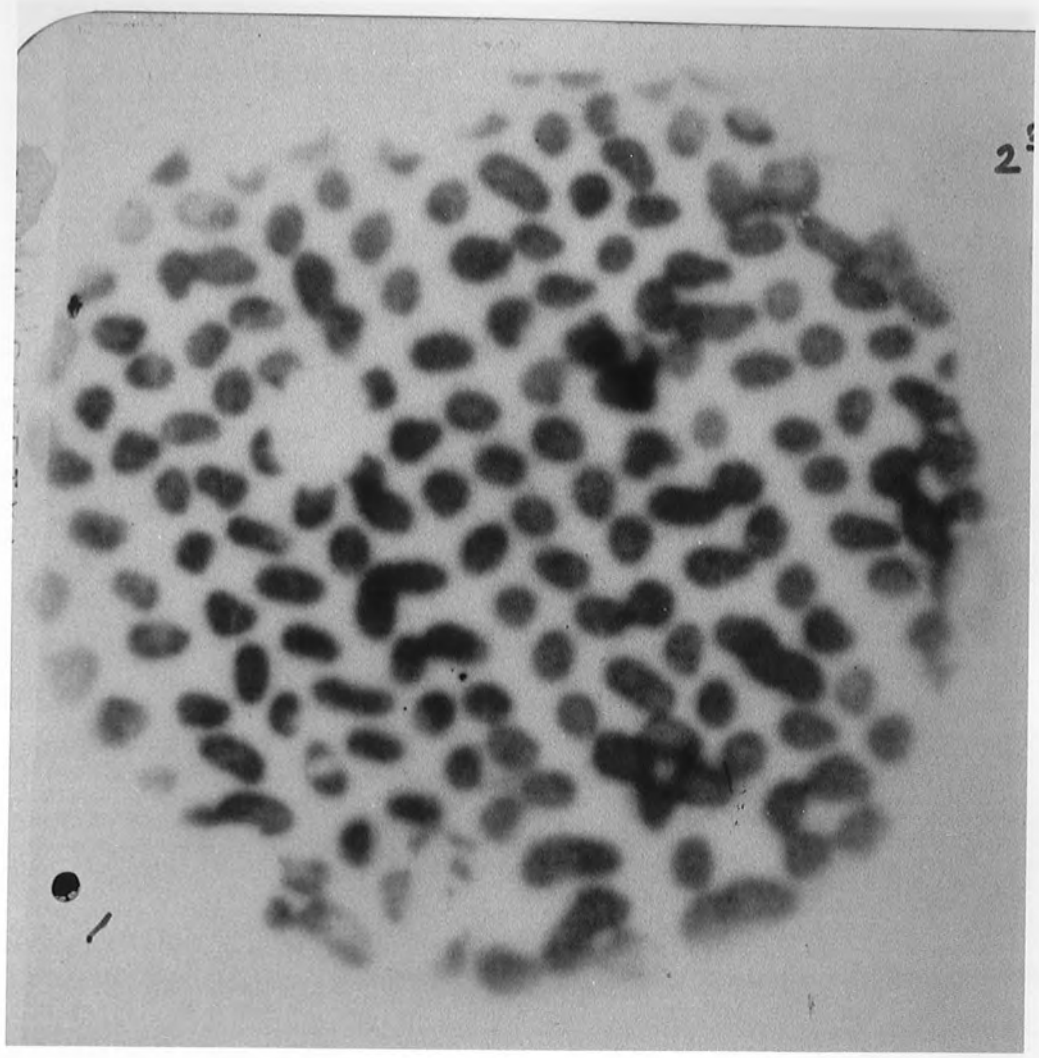


FIG.3.2.5

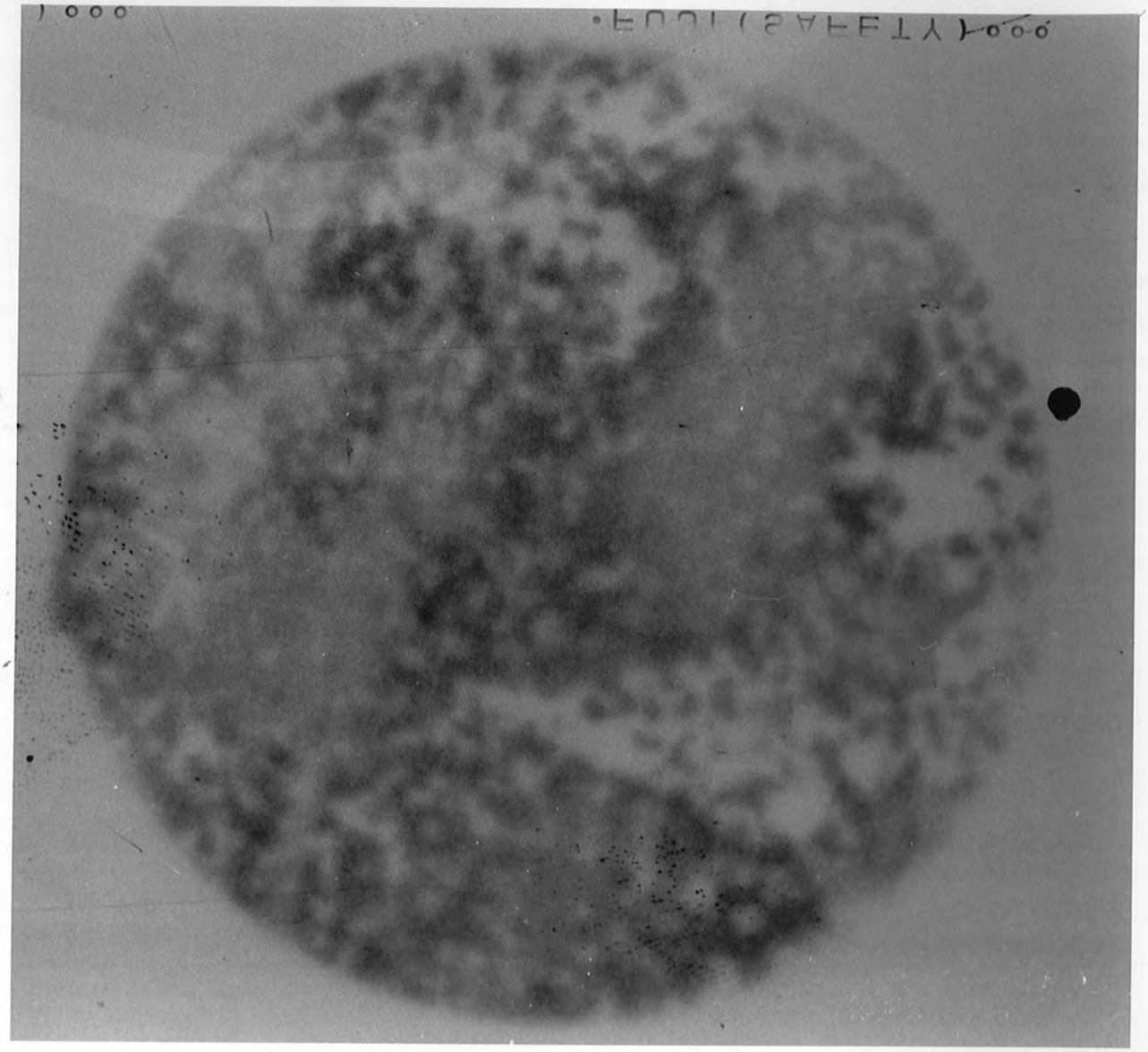


FIG.3.2.6

(i) to introduce into the Δ vectors, and

(ii) to generate a hybridisation probe, by nick-translation using a ^{32}P labell and DNA polymerase I (Section 2.2.5), which could used later in the experiment to analyses the recombinant plasmids for the vicilin insert.

The Δ vectors and vilicin cDNA fragment were then ligated using T_4 DNA ligase, and transformed into *E.coli JM83*. Ampicilin resistant colonies were then subjected to a colony hybridisation or colony blot hybridisation (picking off colonies onto nitrocellulose or bloting onto nitrocellulose repectively - section 2.2.12). Many colonies were screened from each separate Δ plasmid vectors, and standardised against both positive (vicilin$^+$) and negative (vicilins$^-$) controls. These results were shown in figures 3.2.4 to 3.2.6, except the negative control is not shown as the picture showed non-hybridised proportions with a little background.

The positive colonies were then picked off into L-amp broth and cultured overnight, and the recombinant plasmids collected using a large minipreparation (Section 2.2.9.2). These were then subjected to restriction analysis. Figure 3.2.3 show the results of such an analysis, the putative recombinant plasmids; pL1 corresponding to pYSV9 no deletion plus vicilin cDNA insert, pL2 to pYSV9 Δ1 plus insert, pL3 to Δ7 plus insert, pL4 to Δ23 plus insert, pL5 to Δ26 plus insert, and pL6 to Δ27 plus insert. The agarose gel was also subjected to a Southern blot to analyse the recombinant plasmids, although the result of this blot is not shown, the blot did show that the recombinant plasmids contained

the vicilin. It this stage is was not known wether or not the vicilin was in the correct orientation for expression.

To determine the orientation of the inserted vicilin fragment the recombinant plasmids pL1 to 6 were restricted with either the restriction endonucleases EcoRI and Bgl II, or EcoRI and HindIII; the restriction patterns were then analysed on a 1.75% agarose gel.

EcoRI / Bgl II:

Correct orientation:

pL1 to 6 would give 5 bands of 4.200, 3.355, 2.000, 1.061, and 0.155 Kb,

Incorrect orientation:

pL1 to 6 would again give 5 binds of 4.200, 3.355, 2.000, 1.131 and 0.045 Kb.

EcoRI / HindIII:

Correct orientation:

as incorrect orientation EcoRI/Bgl II,

Incorrect orientation:

as correct orientation EcoRI/Bgl II.

except pL1, this has the HindIII site intact compared with the Δ vectors. Tis would produce 6 bands;

Correct orientation:

i.e. 4.200, 3.355, 2.000, 1.000, 0.155 and 0.061 Kb.

Incorrect orientation:

i.e. 4.200, 3.355, 2.000, 1.000, 0.131 and 0.045 Kb.

The lower molecular weight bands were difficult to see.

These were run against pBR322 restricted with Hae III (See appendices ?) which has 22 sites fot Hae III restiction, but only 8 of the larger fragments were seen, λ DNA restricted with HindIII or EcoRI (See appendix A), and pYSV9 restricted with EcoRI and/or HindIII (See appendix B).

The correctly orientated recombinant plasmids were then to be introduced into the yeast *S. cerevisiae*, and analysis of the possible expression determined.

The transformation of the plasmid pDUB2018 into *S. cerevisiae* was acheived later and the results noted (Section 3.3 and 3.4).

3.2.3 Discussion.

The production of the recombinant plasmids was acheived, but the analysis of the expression in yeast of these plasmids was not. This was only due to the limited time available to the project. If the Δ plasmids were sequenced and the expression of the inserted vicilin cDNA analysed the results of the deletions on the α mating factor reading frame would have been seen.

The plasmid pYSV9 (Figure 3.2.1) was primarily obtained from the Leicester Biocentre, it was made from the 5' portion of the *MFα1* gene obtained from a plasmid provided by Dr. I. Herskowitz. The 2μm-*LEU 2* portion of the