PUTATIVE PROMOTER SEQUENCES FOR DIFFERENTIAL EXPRESSION DURING WINE FERMENTATIONS

by

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In memory of
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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where reference has been made in the text.

If accepted for the award of Doctor of Philosophy, this thesis will be available for loan and photocopying.

Renata Martina Polotnianka

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ABBREVIATIONS

A	:	adenine
aa	:	amino acid
asn	:	asparagine
bp	:	base pair
C	:	cytesine
cDNA	:	complementary deoxyribonucleic acid
Ci	:	Curie
cmp	:	counts per minute
DAP	:	diammonium phosphate
dATP	:	deoxy-adenosine triphosphate
dCTP	:	deoxy-cytosine triphosphate
dGTP	:	deoxy-guanine triphosphate
dNTP	:	deoxy-ribonucleotide triphosphate
dTTP	:	deoxy-thymidine triphosphate
G	:	guanine
kb	:	kilo base
OD	:	optical density
poly A	:	polyadenylic acid
Pu	:	purine
Py	:	pyrimidine
RNase	:	ribonuclease
rp	:	ribosomal protein
rpm	:	revolutions per minute
ser	:	serine
T	:	thymidine
thr	:	threonine
UV	:	ultra violet

SUMMARY

Genetic manipulation is now recognised by the wine industry as a means of developing new yeast strains. However, the expression of a new gene must be compatible with oenological conditions, which in general do not allow the construction of expression vectors with commonly used promoters. New promoters must be identified that suit these growing conditions. To this end, gene expression of wine yeast under grape juice fermentation conditions was studied.

Initially, microvinification trials were conducted to monitor yeast growth and fermentation parameters and study gene expression profiles. *In vitro* translation experiments detected changes in gene expression in response to the changing fermentation environment. Subsequent research aimed to isolate the differentially expressed sequences.

Complementary DNA (cDNA) libraries were prepared from yeast cells harvested at early (exponential) and late (stationary) stages of growth during the anaerobic fermentation of grape juice. Differential screening of these libraries identified clones which represent genes expressed at specific stages (non-constitutive) of a wine fermentation. Of 281 clonal isolates, 144 clones were classified as "early" stage-specific and 137 as "late" stage-specific. The variety of genes isolated and an approximation of their expression levels was assessed by sequence analysis and by cross-hybridisation between clones.

Hybridisation analysis of RNA from an anaerobic ferment was used to demonstrate the stage-specific expression of the clones. In general, "early" stage-specific clones were strongly expressed during the exponential growth phase but not during stationary and cell decline phases. "Late" stage-specific clones were not expressed during the growth phase but were strongly induced from early stationary phase with expression persisting throughout stationary phase.

A distinct relationship was observed between expression and the yeast growth phases. Consequently, experiments were also conducted in order to define whether the differentially expressed genes isolated were casually associated with the growth cycle in yeast or were expressed as a result of the fermentation conditions. Oxygen, ethanol and sugar concentrations were altered at specific times during wine fermentations and RNA was extracted. RNA hybridisation analysis indicated that the induction or repression of the majority of clones tested was coordinately adjusted with the change in cellular growth rate. Although, some quantitative changes in the level of expression of a few clones were also observed, indicating a metabolic response to the altered environmental conditions.

On the basis of their expression patterns two cDNA clones were chosen for detailed characterisation and isolation of the promoter sequences of the corresponding genes. DNA sequence analysis identified the "early" stage-specific clone as containing a ribosomal protein gene (*L45*). The "late" stage-specific clone showed some sequence similarities to a yeast cell wall protein. A genomic library was constructed and the 5' non-coding region for each gene isolated.

An integrative vector system, employing the *Escherichia coli* β-glucuronidase (*uidA*) gene as a reporter for expression analysis, was designed and constructed to test the ability of the isolated sequences to act as functional promoters under fermentation conditions. Vectors containing isolated putative promoter sequences in sense and nonsense orientations were transformed into a polyploid wine yeast strain. Integration was targeted to the *ILV2* locus. DNA hybridisation analysis of transformants confirmed vector integration, although not into every copy of the *ILV2* gene of the polyploid yeast strain.

Transformed wine yeast colonies were observed by GUS plate assay to be expressing the β-glucuronidase gene. Transformants were used as pure cultures for inoculation of wine fermentations. Cell suspension assays for the presence of β-glucuronidase were conducted in cells extracted from the ferment and RNA hybridisation analysis was also carried out. Little β-glucuronidase mRNA was detected by hybridisation analysis, however, GUS activity was observed in the cell suspension assays indicating that some translation of β-glucuronidase mRNA had occurred. In all assays little or no β-glucuronidase enzyme was observed in transformants with putative promoter sequences in the inverted orientation.

Thus, this thesis describes the isolation of sequences that can produce differential expression of a gene during anaerobic wine fermentations, the use of these sequences in the development of expression vectors and the application of this work to the production of genetically engineered wine yeasts for commercial purposes.

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CHAPTER 1 : GENERAL INTRODUCTION AND PROJECT AIMS

Traditionally, wines have been produced by natural fermentation caused by the occurrence of yeasts originating from the grapes and wineries. No deliberate inoculation was made to initiate the fermentation. However, since the introduction of pure cultures by Hansen (1888) into the beer industry, many wine makers now also use pure yeast cultures. This practice enables specific strain characteristics to be utilised in the production of a wide range of wines and also provides greater reliability and control of fermentation resulting in wines with fewer flavour defects.

With the introduction of pure culture inoculation of wine yeast into wine-making, many technical advances in wine fermentation have also followed. The unreliable trial-and-error methods have been replaced increasingly by planned scientific improvement programs. To date however, most wine-improvement research has concentrated on the improvement of grape varieties and wine making practices. In the future, increasingly sophisticated and controlled fermentation technologies will demand strains more highly tailored to specific functions.

Such demands will include the modification of traditional wine yeast strains to reduce process costs, enhance wine flavour and quality, improve aspects of process efficiency, develop new products and increase the value of spent yeast. It may not be possible to develop a single "best strain", yet a number of strains with different characteristics, all of which will perform suitably under specific conditions, are attainable.

To this end, it is important to define the most desirable characteristics of wine yeast strains and the requirements of the wine industry in genetic terms - to select specific targets for modification and scientific research. Reviews and discussion of desirable yeast properties and the means by which they may be modified have been undertaken by many researchers (Thornton, 1983; Snow, 1983;. Spencer and Spencer, 1983; Sturley and Young, 1986; Yap, 1987; Henschke, 1989; Thornton and Bunker, 1989; Pretorius and van der Westhuizen, 1991; Barre *et al.*, 1993).

Attempts to develop improved industrial yeast strains (*Saccharomyces cerevisiae*) by traditional genetic techniques (mutation, hybridisation, rare mating and spheroplast fusion) have had limited success (Sturley and Young, 1986). However, in recent years the application of recombinant DNA techniques has added a new momentum to commercial yeast improvement.

One aim of the Australian wine industry is to establish a system for the genetic engineering of wine yeast strains which will be suitable for industrial application. The following criteria are considered most important in achieving this aim: an efficient transformation method for the introduction of foreign DNA into wine yeast; demonstration that the foreign DNA does not adversely affect the fermentation characteristics of the yeast strain; stability of the foreign DNA in the yeast population throughout the fermentation; appropriate expression of the foreign gene in the yeast cell and; isolation of signal sequences to ensure secretion of the foreign gene product.

Research in this area has led to the efficient introduction of foreign DNA into wine yeast using an integrative vector targeted to the *ILV2* locus. The introduced DNA showed stable inheritance without obvious affects on the fermentation performance of the strain. Selection of transformed yeast cells was based on a dominant marker which conferred resistance to the herbicide, sulfometuron methyl (Petering *et al.*, 1991a/b).

Although addition of new genetic material may be beneficial in the desired area, it may also have deleterious effects in another. Genes altered for fermentation efficiency may change wine flavour and aroma by altering the normal concentration of yeast metabolites produced. Also, as high expression levels of the cloned genes are often desired, their expression may stress the cells. This can result in lower growth rates as well as reduced overall productivity and competitive ability. Therefore, for the system to have industrial applications, mechanisms for the controlled expression of newly introduced genes must be established.

There have been extensive studies of gene expression in *Saccharomyces cerevisiae* since the advent of genetic engineering and the new techniques of molecular biology. Two basic promoter types are commonly used to express foreign genes in yeast: constitutive promoters which allow a gene product to be synthesised continuously or regulatable promoters which are positively or negatively regulated to induce or repress transcription. Regulated promoters offer greater flexibility than constitutive promoters and allow the gene product to be expressed only in response to defined conditions. In laboratory yeast strains, regulatable promoter studies have focused mainly on genes regulated in response to external environmental cues, such as heat shock (Lindquist, 1981; Lindquist and Craig, 1988; Susek and Lindquist, 1990; Nover, 1991), exogenously added carbon sources (St. John and Davis, 1981a/b; Moore *et al.*, 1991) and amino acid starvation (Struhl 1982a/b; Struhl and Hill, 1987; Hinnebusch, 1988). However, such promoters are often inapplicable for use in alcoholic fermentations because of the original high sugar

concentration, restraints concerning product composition and limitations associated with the fermentation procedure and the use of additives. Consequently, much of the fundamental research involved in gene expression is not directly applicable to wine yeast under fermentation conditions. Growth in this complex medium is likely to involve a wide range of regulatory controls affecting gene expression. To this end, research was undertaken to study gene expression during fermentations of grape must.

The ultimate aim of this project was to identify effective promoters to regulate gene expression for optimal gene product activity during wine fermentations. In terms of the wine industry, this concerns the development of modules for gene regulation, that depend on parameters compatible with oenology. Consequently, identification of genes that were differentially expressed during wine fermentations and isolation of their promoter sequences was central to this project. Further experiments were designed to examine the major regulatory parameters of a wine fermentation to determine their effects in the induction of gene expression and on transcript levels. As more is understood about the mechanisms used to regulate gene expression from promoters in wine yeast, greater sophistication in the design of economic strategies for foreign gene expression in wine yeast will result.

CHAPTER 2 : LITERATURE REVIEW

2.1 GENERAL YEAST FEATURES

The original *Saccharomyces* wine yeast strains were derived from the population of yeasts that occur naturally on the grape skins. These yeast strains were initially characterised taxonomically into at least 29 different species or varieties (Lodder, 1970; Kunkee and Goswell, 1977). Most of these species were separated primarily on the basis of their sugar fermentation and assimilation patterns. Subsequently, they have been reclassified as *Saccharomyces cerevisiae* (Kreger van Rij, 1984). Importantly, not all yeast belonging to this species are capable of producing palatable wine. Included within this species are laboratory and industrial (distilling, brewing, wine and baking) yeast strains. A brief outline of the genetics of *S. cerevisiae* will be described below, particularly as it relates to differences between strains of industrial and laboratory yeast.

Genetic studies with *S. cerevisiae* were pioneered by Winge (1935) who first observed haploid and diploid phases in the yeast life-cycle. These studies were extended by Lindegren and others, who further unravelled details of the life-cycle and identified two opposite mating types (Lindegren, 1943; Winge and Roberts, 1958; Mortimer and Hawthorne, 1969).

The laboratory strains of *S. cerevisiae*, usually occur in a stable haploid state, termed heterothallic, and exist as one of two mating types (Mata or Matα). Diploids are formed when the cell of one mating type comes into contact with a cell of another mating type. In contrast, strains in which cell fusion and diploid formation occur among cells derived from a single spore are termed homothallic. The genetic basis for homothallism has been shown to be a single gene, of which the dominant allele confers homothallism (*HO*) and the recessive allele confers heterothallism (Harashima *et al.*, 1974). The presence of the *HO* gene brings about a high frequency of switching between mating types during vegetative growth (Hicks and Herskowitz, 1976; Herskowitz and Oshima, 1981). The surveys that have been made of wine yeast indicate that they are typically homothallic (Thornton and Eschenbruch, 1976; Snow, 1979; Kusewicz and Johnston, 1980).

Unlike haploid laboratory yeast strains, which have been subjected to constant selection for regular sexual reproduction and high spore viability, industrial yeast are invariably diploid or polyploid, they lack a specific mating type, are reticent to mate and exhibit low spore viability (Johnston, 1965; Fowell, 1969; Anderson and Martin, 1975). It has been

suggested that these properties confer a measure of phenotypic stability which makes them less susceptible to the destabilising forces of mutation and recombination (Stewart, 1981; Kielland-Brandt *et al.*, 1983; Panchal *et al.*, 1986).

Laboratory strains of yeast have become useful organisms in which to undertake genetic and molecular analysis of basic biological processes. This stems from their ease of manipulation, excellent genetics and their high efficiency of homologous recombination. Industrial yeasts are very different. *Saccharomyces* used in the production of alcoholic beverages differ from the laboratory strains on which knowledge of yeast genetics has been based. Their polyploid state makes analyses of important industrial traits more difficult and complicates the genetic improvement of these yeast strains.

2.2 GENETIC TECHNIQUES FOR STRAIN DEVELOPMENT

Saccharomyces cerevisiae can be manipulated genetically in many ways. Whereas some techniques alter limited regions of the genome, others are used to recombine or rearrange the entire genome. Past techniques for the genetic programming of industrial yeast strains were clonal selection of variants, mutation and selection, hybridisation, rare mating and spheroplast fusion. However, these techniques lack the specificity required to modify yeasts in a well controlled way. On the other hand, the emergence of recombinant DNA technology has provided the opportunity to specifically alter single characteristics in industrial yeast strains in a defined manner. All of the above methods have been used to create new industrial strains and will be described in the following sections.

2.2.1 Classical Genetic Techniques

2.2.1a Clonal and Mutagen Selection

The clonal selection procedure requires the screening of a large number of single cells derived from a parental strain for a particular characteristic. This method has been used to select non-foaming wine yeast mutants (Ouchi and Akiyama, 1971; Eschenbruch and Rassell, 1975); strains with improved ethanol tolerance (Brown and Oliver, 1982); and those with reduced hydrogen sulphide production (Rupela and Tauro, 1984). Clonal selection takes advantage of the natural genetic heterogeneity in wine yeast strains. However, as natural variation may be limited, the application of mutagenesis can be used to extend the pre-existing diversity.

Selection after mutagen treatment has been used in the improvement of wine, brewing, baking and distillers yeast. Mutants of brewing yeasts with increased flocculation and with modified abilities to produce diacetyl and hydrogen sulphide were isolated by Molzahn (1977). Rous and Snow (1983) mutagenised derivatives of a single spore isolate of wine yeast to lower the fusel oil content of wine and distilled beverages. They obtained a leucine requiring mutant that reduced the concentration of isoamylalcohol in wine by 50 percent. More recently, experiments by Kunkee *et al.* (1987) have shown that brandy produced with this yeast strain was equally lower in fusel oil content.

Mutagenesis has the potential to disrupt or eliminate undesirable characteristics and to enhance favourable properties in industrial yeasts, however, this process is limited as the mutagen treatment often produces additional mutations to the one of interest and these may be undesirable.

2.2.1b Hybridisation, Rare Mating and Spheroplast Fusion

New genetic combinations may be achieved by the production of hybrids by conventional crossbreeding. This involves the fusion of haploids of opposite mating types to yield a heterozygous diploid. Recombinant progeny are recovered by sporulating the diploid and recovering the haploid ascospores. Backcrossing of the derived strain to one of its original parents is done to remove undesirable characteristics. Crossbreeding has been used to introduce the killer characteristic into sake (Ouchi and Akiyama, 1976) and wine (Hara *et al.*, 1980) yeast, as well as, to produce a flocculent non-foaming wine yeast strain with high ethanol production and a high fermentation rate (Romano *et al.*, 1985).

Hybridisation has been an extremely useful tool for the study of yeast genetics. The value of this technique for the construction of new industrial strains, however is rather limited because of the reluctance of commercial yeast strains to mate. Consequently, two alternative "hybrid" techniques have been developed to extend the range of crosses that can be performed.

Rare mating is a way of forcing hybridisation between a non-mating polyploid strain and a haploid donor strain carrying desirable characteristics. Rare mating can be used to construct "true" hybrids in which nuclear and cytoplasmic characters are introduced from the donor strain, or for cytoduction, a rare form of hybridisation whereby cytoplasmic genetic elements, or just one or two nuclear chromosomes are transferred into the industrial strain.

Hybrid brewing yeast have been obtained by rare mating involving crosses between auxotrophic laboratory strains and respiratory-deficient (Tubb *et al.*, 1981) or antibiotic resistant, mitochondrial mutant (Spencer and Spencer, 1977) strains of brewing yeast. Killer yeasts, which show characteristics that are close to their fermenting parental strain have also been constructed for both the beer (Young, 1981; Hammond and Eckersley, 1984) and wine (van der Westhuizen and Pretorious, 1989) industries. In another experiment, Thornton (1985) mated the spores of a non-flocculent wine yeast with haploid cells of a heterothallic, flocculent laboratory strain. Five successive back breedings of the diploid hybrids with spores of the wine yeast resulted in a flocculent wine yeast strain. A flocculent strain has also been obtained by the method of cytoduction, with the transfer of a single chromosome from a FLO5 laboratory strain to a non-flocculent wine yeast strain (Vezinhet *et al.*, 1992).

Spheroplast fusion is a direct, asexual technique that is used in crossbreeding as a supplement to mating. Spheroplast fusions are achieved by the incomplete enzymatic removal of yeast cell walls and the induction of fusion. This method can produce "hybrids" between non-mating commercial yeast strains and greatly increases the range of yeasts that may be exploited, as inter-specific and inter-generic fusions may be possible. The use of spheroplast fusion to modify industrial yeasts is illustrated by the construction of brewing strains able to ferment dextrins (Freeman, 1981; Russell *et al.*, 1983a), industrial yeast strains with improved tolerance to ethanol (Seki *et al.*, 1983; Legmann and Margalith, 1983) and baker's yeast with added genes for starch utilisation (Bortol *et al.*, 1988).

The problem with all the genetic techniques described thus far, is that they have a limited potential to create new strains with a markedly different genetic character. This is because these procedures are primarily concerned with either the modification and/or abolition of existing genetic traits or the reassortment of a limited pool of genetic variation. In contrast, recombinant DNA technology, coupled with transformation, facilitates both the construction of novel strains by the controlled introduction of a wide variety of new genes and enables the directed modification of existing genetic traits.

2.2.2 Recombinant DNA Technology

Yeast genetic transformation is the addition of exogenous DNA to yeast and its subsequent incorporation into the genetic framework of the cell, resulting in the acquisition of a novel characteristic. This characteristic may arise from the introduction and expression of a new gene or by disrupting existing genes.

The first yeast transformation was conducted by Hinnen *et al.* (1978) who designed a protocol to introduce DNA into cells by enzymatically altering the permeability offered by the cell wall. Since then, alternative protocols involving chemical pretreatments with alkali metal salts (Ito *et al.*, 1983, 1984; Brzobohaty and Kovac, 1986), formation of spheroplasts (Burgers and Percival, 1987) and electroporation (Hashimoto *et al.*, 1985; Delorme, 1989; Simon and McEntee, 1989) have been developed.

Once the DNA is introduced, an important aspect of transformation is the ability to stably maintain the foreign DNA within the host. Consequently, DNA to be introduced into host cells need to be incorporated into a transport vehicle or vector to ensure stable inheritance. In general, two major types of DNA vectors are employed for the introduction of foreign DNA into yeast cells. These are autonomously replicating and integrating vectors. Autonomously replicating plasmids contain sequences which allow them to replicate independently of the nuclear genome. They are usually found in high copy number within a host but are generally mitotically unstable. Integrating vectors involve site-specific integration of the vector into chromosomal DNA mediated by homologous recombination, resulting in high heritable stability at low gene dosage.

These fundamental plasmid frameworks have been the basis for subsequent development and enhancement of vector design. For example, improved stability of autonomously replicating plasmids was gained by the cloning of the yeast centromeric region (Clarke and Carbon, 1980). Subsequent addition of telomeric sequences by Murray and Szostak (1983) led to the development of yeast artificial chromosomes (YACs). As a consequence, DNA of several hundred kilobases can now be cloned. For comprehensive information of available yeast vectors and the particular sequences they comprise (some to be discussed below) refer to reviews by Parent *et al.* (1985), Ausabel (1987), Bitter *et al.* (1987) and Rodriguez and Denhardt (1988).

Another feature of yeast vectors, includes sequences which allow selection of the few cells that have incorporated the vector. Common selective sequences for laboratory yeast are prototrophic markers such as *LEU2*, *URA3*, *TRP1* and *HIS3* which complement recessive auxotrophic mutations in the host strain (Beggs, 1978; Struhl and Davis, 1980; Tschumper and Carbon, 1980; Rose *et al.*, 1984). However, dominant selectable markers are generally used for transforming industrial yeast strains due to their polyploid state. Common dominant selectable markers frequently used in the genetic manipulation of industrial strains are hygromycin B resistance encoded by a bacterial phosphotransferase gene (*HPH*) (Gritz and Davies, 1983; Kaster *et al.*, 1984), the G418 antibiotic resistance

gene (Webster and Dickson, 1983) and the chloramphenicol resistance gene (*CAT*) (Cohen *et al.*, 1980; Hadfield *et al.*, 1986) encoded by *E. coli* transposons (Jimenez and Davies, 1980; Webster and Dickson, 1983), as well as the copper resistance gene (*CUP1*) of *Saccharomyces* (Fogel and Welch, 1982; Butt *et al.*, 1984; Henderson *et al.*, 1985; Enari *et al.*, 1987).

The vector may also contain various DNA sequences and signals which regulate the expression and processing of a foreign DNA sequence into a protein: known as the "expression complex" it comprises of a promoter and terminator and sometimes signal sequences for protein secretion.

In general, two types of promoters, constitutive or regulatory, can be used to express a foreign gene in yeast. Constitutive promoters allow a gene product to be synthesised continuously throughout the culture period. Regulated promoters are acted on positively or negatively by regulatory proteins, which define the cellular condition, to induce or repress transcription.

Early expression work focused on the use of strong constitutive promoters (particularly those derived from the yeast glycolytic genes) to produce high levels of product. Examples are: alcohol dehydrogenase *ADH1* (Hitzeman *et al.*, 1981; Bennetzen and Hall, 1982a), phosphoglycerate kinase *PGK* (Dobson *et al.*, 1982; Tuite *et al.*, 1982) and glyceraldehyde-3-phosphate dehydrogenase *GAPDH* (Holland and Holland, 1980; Bitter and Egan, 1984). For further information, Shuster (1989) has reviewed the use of these glycolytic promoters in the expression of heterologous proteins in yeast.

Although aiming for a strong promoter is a frequent goal, the need for promoter control or adjustment of promoter activity can become quite apparent. High expression levels may be desired, however, the synthesis of a cloned gene can place additional stress on the cells. This can result in lower growth rate, yield and can reduce overall productivity. Often the detrimental effects of high expression outweigh the positive effects of increasing the gene transcripts. To reduce the negative effects of cloned gene expression, plasmids with regulatable promoters may be employed.

Amongst the most tightly regulated promoters of *S. cerevisiae* are those of the galactose regulated genes *GAL1*, *GAL7*, and *GAL10*. These genes are involved in the metabolism of galactose. Galactose promoters are strongly repressed in the presence of glucose and can only be expressed following the depletion of glucose (St John and Davis, 1981b).

Other regulated promoters include the promoter of the acid phosphatase gene (*PHO5*) which is regulated by inorganic phosphate concentration (Bostian *et al.*, 1980b; Meyhack *et al.*, 1982) and the alcohol dehydrogenase 2 (*ADH2*) promoter which is tightly repressed in the presence of high glucose and switched on in low glucose (Beier and Young, 1982; Beier *et al.*, 1985).

Although there are significant advantages to using regulatable promoters there may be certain constraints in their use that arise from the nature of the genes to be expressed and the expression conditions. More recently, hybrid promoters have been constructed for the expression of heterologous proteins in yeast, which take advantage of constitutive promoters and their intrinsic strength, as well as regulated promoter elements to control expression of the gene. The validity of this technique was established when Guarente *et al.* (1982) showed that the UAS of the *GAL10* promoter could confer galactose inducibility on the *CYC1* promoter when inserted upstream of the *CYC1* mRNA initiation start site. The hybrid promoter showed inducibility in the presence of galactose but selection studies showed that overall transcription levels from the hybrid were determined by *CYC1* functions. That is, hybrid promoters display the desired properties of each of the promoters used. Other examples of hybrid promoters include: *ADH/GAPDH* (Cousens *et al.*, 1987; DeBaestselier *et al.*, 1991), *GAL/CYC1* (Guarente *et al.*, 1982; Hinchliffe *et al.*, 1987), *PHO5/GAP* (Rink *et al.*, 1984; Hinnen *et al.*, 1989), and a temperature regulated promoter based on the mating type expression system (Sledziewski *et al.*, 1988; Kobayashi *et al.*, 1990).

Yeast transcription terminators are also usually present in expression vectors for efficient mRNA 3' end formation. Transcription termination signals are necessary for termination of transcription, cleavage of the 3' end and subsequent polyadenylation. Terminators from a number of genes have been used in expression vectors: *TRP1* (Hitzeman, 1983), *ADH1* (Urdea *et al.*, 1983) and *GAPDH* (Rosenberg *et al.*, 1984).

Finally, once a sequence has been expressed it may be desirable for the protein to be secreted. This requires the presence of a signal sequence to direct the protein product through the secretory pathway. In secretion vectors, the secretion signal sequence is found downstream to a promoter, preceding the foreign gene to be expressed. Some mammalian proteins have been secreted from yeast using their own signal sequences (Kingsman *et al.*, 1985b). However, it is generally observed that the efficiency of secretion is low and the processing at the signal sequence can be variable (Kingsman *et al.*, 1985a, 1988). Heterologous protein secretion is often more efficient when secretion signals from yeast are used. A number of secretion vectors based on yeast signal sequences have been

constructed. The leader peptide sequence of the α mating pheromone (Bitter *et al.*, 1984; Brake *et al.*, 1984; Lancashire and Wilde, 1987; Ernst, 1988) and the killer toxin of *S. cerevisiae* (Skipper *et al.*, 1985; Tokunga *et al.*, 1988; Cartwright, 1992) have been extensively used in vectors to secrete homologous and heterologous gene products in yeast.

Much of the fundamental research involved in setting up these vector systems was conducted to bring about heterologous expression in laboratory yeasts. Application of this information to industrial needs is now being undertaken. Choice of specific vector sequences is required to meet the economic and logistic factors involved in commercial production. To do this, it is necessary to have a detailed understanding of these sequences and their functions within the yeast cell. The following sections describe the structure of yeast promoters and their signal sequences and the application of recombinant techniques to industrial strains with emphasis and examples of the need for regulated expression.

2.3 ORGANISATION AND EXPRESSION OF THE YEAST GENOME

Saccharomyces cerevisiae has a small compact, nuclear genome of less than 14 mega bases with an estimated total of 6,000 to 8,000 genes (Link and Olson, 1991; Olson, 1991; Oliver, 1996) divided between 16 chromosomes in a haploid strain (Mortimer *et al.*, 1989). Industrial strains are predominantly diploid or polyploid and the precise determination of chromosome number within these strains is unknown.

The chromosomes are located in the nucleus and account for 80 to 85 percent of the total yeast DNA (Petes, 1980). The remainder of DNA is found in the cytoplasm. The major cytoplasmic portion of the genome consists of mitochondrial DNA (Dujon, 1981). Other cytoplasmic genetic elements consist of the 2μm plasmid (Sinclair *et al.*, 1967; Broach, 1981) and in killer strains, double stranded RNA molecules (Tipper and Bostian, 1984; Wickner, 1981, 1983).

The genome of *S. cerevisiae* contains little repetitive DNA and a high percentage of the genome is shown to be gene functional. Renaturation kinetics suggest that 95 percent of the nuclear genome is single copy sequence (Fangman and Zakian, 1981). The protein encoding genes are relatively simple with comparatively few non-coding (intron) regions (Levy, 1994). The most recently published version of the genetic map of *S. cerevisiae* assigns 1046 genes to specific sites, chromosomes and regions (Mortimer, *et al.* 1992).

Generally, many, if not most yeast genes are always transcribed at a basal level under normal growth conditions (St John and Davis, 1979). However, research also indicates that some genes are expressed at variable rates depending on the physiological conditions (St John and Davis, 1981b). Most expression studies to date have focused on those genes whose expression is regulated in response to particular environmental and developmental cues. These cues include regulation as a function of exogenously added carbon sources (St. John and Davis, 1981a/b; Moore *et al.*, 1991), of amino acid starvation (Struhl, 1982a/b; Donahue *et al.*, 1983; Hinnebusch, 1988), of time within the cell division cycle (Nasmyth, 1985; Breeden and Nasmyth, 1987; Nasmyth and Shore, 1987; Breeden, 1988) and of cell mating type (Klar *et al.*, 1981; Abraham *et al.*, 1984; Wilson and Herskowitz, 1986; Hall and Johnston, 1987).

Regulation of gene expression may be achieved at many different levels: overall organisation of genetic material within the chromosomes and in the nucleus, transcriptional steps (initiation, termination), mRNA metabolism (export, splicing, capping stabilisation/degradation) and translational steps (initiation, elongation, termination). However, in most cases, the major adaptive changes in gene expression have been shown to be coordinated by control mechanisms acting at the level of transcription. The next section describes the means by which genes are regulated.

2.4 MOLECULAR REGULATION OF GENE EXPRESSION

Transcription initiation in yeast can be described as a process in which RNA polymerase initiates RNA synthesis at discrete sites.

Yeast RNA polymerases are large multimeric proteins of high molecular weight. There are three different types of RNA polymerase each of which is responsible for the transcription of different classes of genes. RNA polymerase I acts on the clustered genes coding for the large ribosomal RNAs (rRNAs) which form the bulk of the cellular transcripts. RNA polymerase III transcribed genes encode a variety of different RNAs including transfer RNA (tRNA) and small rRNA molecules which together yield much of the remainder of the total yeast RNA. The structure, organisation and regulation of these genes has been reviewed by Sentenac (1985), Murphy *et al.* (1989), Reeder (1990) and Thuriaux and Sentenac (1992). Only a small amount (1 to 5 percent) of total yeast RNA corresponds to the transcription of the protein coding genes which are transcribed by RNA polymerase II (McLaughlin, *et al.*, 1973). The following section will focus on the expression of these class II genes, which essentially encode the messenger RNAs (mRNAs) that are in turn translated into proteins.

Transcription in yeast is controlled mainly, but not exclusively, by sequences found 5' upstream from the coding region. These 5' upstream sequences may extend over several hundreds of base pairs and have been collectively known as "promoters". The following section will give a brief outline of yeast promoters and their function, including transcription initiation, information about the *cis*-acting DNA sequences that constitute a promoter, the proteins that interact with these sequences and the molecules that affect the activity of these proteins such that they influence transcription and regulation. There is a wealth of recent research in this area with extensive reviews by Struhl (1987), Heslot and Gaillardin (1992a) and Mellor (1993). This is in no way then a definitive description but merely the briefest of summaries.

2.4.1 Promoter Structure

Gene promoters have been well described in yeast and can be sub-divided into 2 functional domains which regulate the efficiency and accuracy of the initiation of transcription.

i) transcription initiation site, adjacent to the coding sequence of the structural gene. This site itself may be broken up into 2 regions: the TATA element (a highly conserved sequence, consensus TATAAAA) and the initiator element (I) (where transcription initiation actually takes place).

ii) an upstream sequence possessing a regulatory effect upon the downstream transcriptional initiation site. This regulatory region is complex and binds regulatory proteins whose activity may be controlled by environmental conditions. Three functionally distinct upstream elements have been defined in yeast promoters, upstream activator sites (UASs), upstream repressor sites (URSs) and constitutive promoter elements.

In any yeast promoter, one of each of these elements can be identified, which together are sufficient for properly regulated transcription, although most promoters have a more complex organisation with multiple elements being present. Noticeable common features of yeast promoters are their relatively large size, often containing redundant information, and the variability in the distances separating individual elements. The approximate distance that each promoter element is separated from its neighbours in a "typical" promoter has been defined by Mellor (1993) and displayed below (Figure 2.1).

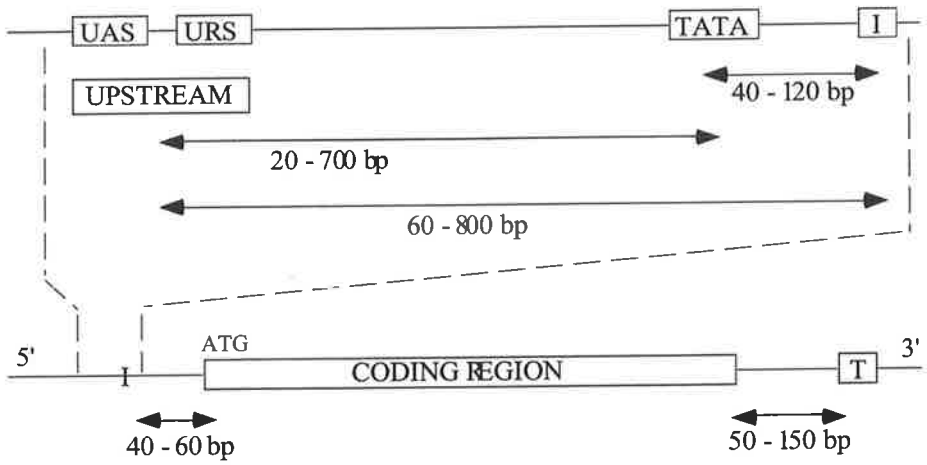


Figure 2.1 : Organisation of a "typical" yeast promoter transcribed by RNA polymerase II. The diagram indicates the typical distances between the three major promoter elements (upstream sequences, the TATA box and the RNA initiation site) and the positioning of the coding region in relation to promoter and termination sequences (Mellor, 1993).

2.4.1a Initiator and TATA Elements

The initiator element, located near the actual initiation start site, is the primary determinant of where transcription begins. It has been shown to be relatively unimportant for determining the rate of transcriptional initiation and has little effect on the overall RNA level (Hahn *et al.*, 1985; Chen and Struhl, 1985). The DNA sequence requirements for yeast initiator elements are poorly understood, although it is clear that many different sequences can carry out this function and often more than one I element is present in any given promoter.

Many yeast promoters have been extensively studied and a number of common features have emerged concerning the initiation sequences. Preferred sequences around the initiation sites, in a group of abundantly expressed yeast genes, were first noted by Dobson *et al.* (1982). RNA initiation was shown to occur at or near a consensus CAAG sequence in all cases. Burke *et al.* (1983) and Healy *et al.* (1987) also observed similarities to the CAAG sequence leading to the creation of a consensus initiation site sequence of PyAAPu. Alternatively, Hahn *et al.* (1985) during a survey of yeast promoters suggested a PuPuPyPuPu initiator start site consensus sequence.

Yeast promoters may contain more than one sequence with homology to the TATA consensus (Faye *et al.*, 1981; Donahue *et al.*, 1982; Guarente and Mason, 1983; Nagawa and Fink, 1985; McNiel and Smith, 1986; Struhl, 1986) which are located at variable distances (between 40bp to 120bp) upstream of the mRNA initiation site. The TATA box

has been shown, in most cases, to be essential for initiation to occur, as deletion mutants of TATA boxes have severely reduced levels of transcription, although regulation is still observed (Struhl, 1982a/b; Guarente and Mason, 1983; Ogden *et al.*, 1986). TATA boxes have also been shown to be orientation specific (Struhl, 1982a/b; Nagawa and Fink, 1985).

In addition to having one or more potential TATA element many yeast promoters contain multiple initiation sites competent for RNA synthesis (Dobson *et al.*, 1982; Ogden *et al.*, 1986; Rudolf and Hinnen, 1987, Healy *et al.*, 1987). Thus, in any given promoter different TATA element / initiator site relationships may exist and a TATA element may potentiate a specific subset of mRNA start sites (Hahn *et al.*, 1985; McNiel and Smith, 1986; Healey *et al.*, 1987). For example, the *CYC1* gene has at least five potential TATA elements (Faye *et al.*, 1981; Hahn *et al.*, 1985; McNiel and Smith, 1986) and the wildtype *CYC1* mRNA initiates at five major sites (Guarente and Mason, 1983; Hahn *et al.*, 1985). However, only two of the TATA elements are required for normal expression. The more upstream element initiates transcription at three sites while the downstream TATA initiates transcription at two distinct sites (Li and Sherman, 1991).

Although there is no precise spacing relationship between the TATA element and initiation sites, there are distance limits over which a TATA/I element can act - called the initiation window (Chen and Struhl, 1985; Hahn *et al.* 1985). The *HIS4* promoter has one I site but four potential TATA boxes only one of which is functional. By moving the TATA element relative to the initiation site, Nagawa and Fink (1985) have shown that the initiation window has a maximum of about 110bp and a minimum of 60bp. When the distance between TATA and initiator elements is increased above or decreased below these parameters, alternative I sites may be used (Chen and Struhl, 1985). It was also shown that destruction of individual TATA boxes by site-directed mutagenesis greatly reduced or abolished the usage of defined I sites (Chen and Struhl, 1985). In addition, deletion of the normal I site results in selection, within the initiation window, of new downstream I elements (Rudolph and Hinnen, 1987).

The mechanism that determines one I element over other related neighbouring sequences is not known, although it is suggested that these sites are defined by an auxiliary sequence known as the determinator (Rathjen and Mellor, 1990) or locator (Maicas and Friesen, 1990). The mechanism by which determinators help in selecting initiation sites remains to be established.

Since different initiation sites within a promoter are utilised coordinately under all conditions the role of multiple TATA/I sites has been shown not to be regulatory (McNiel and Smith, 1986; Healy *et al.*, 1987). However, certain TATAs have been shown to be

functionally distinct by acting in conjunction with one type of upstream element (Struhl, 1986; Mellor *et al.,* 1991). For example, the *HIS3* promoter has one TATA element which acts with a constitutive upstream activating element to mediate constitutive transcription while a second TATA element acts with a UAS to mediate regulated transcription in response to amino acid starvation (Struhl, 1986; Struhl and Hill, 1987).

2.4.1b Upstream Activating Sites

The upstream activator sequence is defined as the element in a yeast promoter at which positive action and regulation of transcription occurs. These UASs interact with regulatory proteins encoded elsewhere in the yeast genome to stimulate transcription. Generally, deletion of the region that defines the UAS results in a significant drop in transcription and the loss of responsiveness to regulatory stimuli (Guarente and Ptashne, 1981; Struhl, 1981, 1982a/b; Guarente and Mason, 1983; Guarente and Hoar, 1984).

The upstream activating elements for a number of different genes have been defined in functional terms and localised to small regions. A comparison of the nucleotide sequences within these regions shows that the elements for different genes do not generally share common sequences and they require different trans-acting factors (regulatory proteins). For comprehensive reviews of UASs and their function refer to Guarente (1984), Struhl (1987) and Guarente (1988).

Upstream activator sequences were first recognised in the early 80's by 5' deletion analyses of the *CYC1* and *HIS3* promoters (Guarente and Ptashne, 1981; Struhl, 1981, 1982a/b; Guarente and Mason, 1983; Guarente and Hoar, 1984). Since then, such elements have been found, via deletion experiments, in a number of yeast promoters, for example; *GAL1-10* (St John and Davis, 1981b; West *et al.*, 1984), *HIS4* (Donahue *et al.*, 1982), *PGK1* (Ogden *et al.*, 1986), *PHO5* (Nakao *et al.*, 1986), *SUC2* (Sarokin and Carlson, 1986) and *CYC7* (Wright and Zitomer, 1984).

Not only do these UASs control the genes in which they naturally occur but they have been found to be exchangeable. Substitution of one UAS for another in a promoter does not alter the transcription initiation site but does alter the expression profile of the hybrid promoter to that from which the substitutive UAS was derived (Guarente *et al.*, 1982; Hahn *et al.*, 1985). These experiments have also shown that UASs may act at variable distances from the TATA site and position is relatively unimportant for promoter function of the constructs. Also, some UAS elements were shown to act bidirectionally as they functioned even when inverted (Guarente and Hoar, 1984; Sarokin and Carlson, 1986).

Although upstream activation at the UAS by trans-acting proteins is likely to be a basic model of gene regulation in yeast their activity may be modulated by URSs when they are in the same promoter area.

2.4.1c Upstream Repressing Sites

The URSs have a negative influence on transcription and respond to specific regulatory signals to lower the efficiency of transcription. The relative position of URS to other upstream sequences is variable. In some cases the negative element is 5' to the UAS (Wright and Zitomer, 1984) although the majority appear to lie between the UAS and the TATA element (Kim *et al.*, 1986; Wilson and Herskowitz, 1986; Mehta and Smith, 1989). Deletion of these sequences has been shown to sometimes result in increased transcription or inappropriate expression (West *et al.*, 1984; Wright and Zitomer, 1985).

Upstream Repressing Sequences interact with trans-acting proteins to regulate the activity of activator sequences such that transcription is repressed. This may occur by displacing trans-acting factors at the UAS or by preventing the interaction between transcriptional activator complexes and RNA polymerase II (Brent, 1985; Struhl, 1985b; Kronstad *et al.*, 1987). Regulation of cell mating type in yeast provides an example of both mechanisms of negative control and has been reviewed by Walton and Yarranton (1989).

Negative control sites have also been identified in the gene encoding glucose repressible alcohol dehydrogenase (*ADH2*) (Beier and Young, 1982; Russell *et al.*, 1983b), the arginase encoding (*CAR1*) gene which is subject to nitrogen catabolite repression (Sumrada and Cooper, 1982), an anaerobic gene (*ANB1*) negatively regulated by oxygen and heme (Mehta and Smith, 1989), a meiosis-specific glucoamylase gene (*SGA1*) (Kihara *et al.*, 1991), as well as the *CYC7* (Wright and Zitomer, 1984, 1985), *TRP1* (Kim *et al.*, 1986) and *STE6* (Wilson and Herskowitz, 1986) genes.

2.4.1d Regulatory Proteins

Upstream sequences act as binding sites for the specific DNA binding regulatory proteins that actually activate or repress transcription. These yeast regulatory proteins were first identified by mutations in unlinked genes, whose products act in *trans* at the upstream sequence of the gene they regulate, to alter their transcription. Overviews of the regulatory DNA binding proteins so far identified in yeast have been assembled by Guarente (1987), Johnson and McKnight (1989), and Verdier (1990).

In general, regulatory proteins are endowed with 3 activities; binding to specific DNA sequences, activation or repression of transcription, and response to specific regulatory signals. Regions responsible for each of these 3 activities have been localised to discrete regions of the polypeptides. For example, DNA binding domains are located in regions of the protein where there is a preponderance of basic amino acids, whereas transcriptional activator domains are found in the most acidic regions of the protein (Brent and Ptashne, 1985; Hope and Struhl, 1986; Ma and Ptashne, 1987; Gill and Ptashne, 1987). These acidic activation regions are likely to be target sites at which protein-protein contacts are made to form an initiation complex before transcription. In some situations they may also be target sites which are modified in some way by regulatory proteins that result in the repression of transcription (Ptashne, 1988).

2.4.1e Regulation

Transcription initiation for protein synthesis in yeast involves the initiation of mRNA synthesis by RNA polymerase II. The available evidence suggests that the polymerase does not recognise specific sequences on the promoter, but rather a complex of transcription factors, comprising of activator proteins and general transcription factors (such as TATA binding proteins), assembled on a DNA scaffold.

The yeast TATA binding protein (TBP) is a simple monomeric, highly conserved but species specific component of the RNA transcription machinery that binds specifically to the TATA element (Buratowski *et al.*, 1988; Sawadogo and Sentenac, 1990). The TBP is critical for all promoter recognition and it is the TBP associated factors (TAFs) that give the protein distinct specificities. The binding of the TBP-TAF complex to the TATA element is one of the initial steps in promoter activation, and mediates the assembly of other general transcription factors, TFIIA, TFIIB, TFIIE and RNA polymerase II into an active transcription complex (Buratowski *et al.*, 1989).

Upstream sequences that bind regulatory proteins to mediate transcription are often hundreds of base pairs away from the initiation complex. Yet it is believed that direct contact of the regulatory protein with the initiation complex must occur for transcriptional activation. Therefore, several models to explain how these sequences bridge the distances to regulate transcription have been put forward. One hypothesis suggests that the activators alter chromatin structure in some way to render the TATA/mRNA initiator region accessible to the transcription complex and RNA polymerase. An alternative hypothesis, which is gradually becoming favoured, is that activators bound at the UAS and the transcription complex touch each other by looping out the intervening sequences. For further information on the molecular mechanisms of transcription regulation in yeast refer to the reviews by Struhl (1987, 1989).

2.4.1f Constitutive Expression

Although most genes studied have been chosen because their expression is regulated in some interesting manner it is likely that many genes are expressed constitutively at low levels under normal growth conditions. Indeed, analysis indicates that most genes are transcribed at similar levels and moreover, in any particular regulatory situation, transcription and translation levels are altered for only a very low percentage of genes (Heresford and Rosbash, 1977; St John and Davis, 1979; St John and Davis, 1981a).

It was hypothesised that constitutive expression of otherwise unrelated genes is achieved by a common mechanism, that is, the upstream elements of their promoters would contain similar DNA sequences. Subsequently, Struhl (1985a) analysed promoter sequences of the constitutively expressed *HIS3*, *PET56* and *DED1* genes in order to determine the DNA sequences necessary for wildtype levels of transcription. The results indicated that constitutive transcription of all three genes depends on poly(dA-dT) sequences upstream of the TATA element. Indeed, the frequent identification of poly(dA-dT) tracts within randomly cloned yeast promoters is consistent with the hypothesis that these elements play an essential role in transcriptional activation (Santangelo *et al.*, 1988).

Poly(dA-dT) sequences may act bidirectionally and their length appears to affect the efficiency of transcription (Struhl, 1985a; Kim *et al.*, 1988). Struhl (1985a) showed that the 17bp poly(dA-dT) region between the *HIS3* and *PET56* genes serves as the promoter element for both and that a higher transcription level is observed in the *DED1* gene due to a longer poly(dA-dT) region.

Promoters containing a constitutive poly(dA-dT) element may also contain additional upstream regulatory elements. This allows the promoter to respond to certain growth conditions by increasing transcription above basal levels. For example, Struhl (1982a/b) showed that the poly(dA-dT) stretch of the *HIS3* gene promoter is distinct from the regulatory sequences that induce *HIS3* expression in response to amino acid starvation.

There are two possible ways that poly(dA-dT) sequences can act to influence transcription. Firstly, the tracts may function by association with a poly(dA-dT) specific transcription factor analogous to the regulated upstream elements in yeast promoters. However, as yet, no dA-dT specific transcription factor has been found. An alternative hypothesis favoured by Kunkel and Martinson (1981) and Prunell (1982) suggests that poly(dA-dT) tracts function as constitutive promoter elements by excluding nucleosomes, thus affecting chromatin structure. This is supported by virtue of the unusual structure (they can form kinks or bends) adopted by dA-dT tracts in solution (Marini *et al.* 1982; Koo *et al.*, 1986; Ryder *et al.*, 1986).

2.4.1g Downstream Sequences

Examples in the literature also support the idea that other regions of a gene can influence the overall level of transcription (Thiele and Hamer, 1986; Chen and Struhl, 1985). One such region, known as the downstream activator sequence (DAS), has been characterised in the coding regions of 3 genes (*PGK*, Mellor *et al.*, 1987; *PYK*, Purvis *et al.* 1987; *LPD1*, Zaman *et al.*, 1992). A sequence of similar nature has also been found in the yeast transposon *Ty* (Warmington and Oliver, 1988). Mellor *et al.* (1987) showed that deletion of such sequences, from the *PGK* gene, caused a tenfold drop in efficiency of transcription. However, it is not known how these sequences act to influence transcription efficiency.

Another downstream sequence important in the efficient transcription of a gene is the domain required for transcription termination and polyadenylation, this will be discussed in the following section.

2.4.2 RNA Stability and Processing

The primary RNA transcript undergoes a number of processing steps before it is exported from the nucleus as mature mRNA. These include, the addition of a 7-methylguanosine cap at the 5' terminus and the excision of introns when present. Also 3' to the translated region the primary transcript is cleaved at a conserved transcription termination site and a number of adenylic acid residues are added to form a polyA tail.

Yeast mRNA half-lives range from about 1 to over 100 minutes (reviewed by Brown, 1989a/b). Several reports suggest that the stability of individual mRNAs is related to basic mRNA properties such as size (Santiago *et al.*, 1986), its 5' cap (Sripati, *et al.*, 1976; Piper *et al.*, 1987) and poly(A) tail length (Littauer and Soreq, 1982; Bernstein *et al.*, 1989; Santiago *et al.*, 1987). Therefore, the sequences that terminate transcription and direct 3' polyadenylation or 5' capping are also important in the control of protein production.

As the transcriptional initiation start site varies from gene to gene it is difficult to draw any conclusion about the structure of the yeast capping site. Nogi and Fukasawa (1983) discovered a canonical capping sequence ACAGTT at the transcriptional start site of *GAL7*, however this sequence has not been found in any other yeast genes. The capping process involves the addition of a methylated guanosine residue (m^7G) via a 5' to 5' phosphodiester bond (Sripati *et al.*, 1976). This modification is catalysed by the yeast nuclear enzyme mRNA guanylyltransferase (Itoh *et al.*, 1987).

Transcription termination in most eukaryotes is brought about by a highly conserved sequence element AAUAAA located 10 to 30 bases upstream from the polyadenylation site (Proudfoot and Brownlee, 1974). It is required for endonucleolytic cleavage as well as polyA addition (Fitzgerald and Shenk, 1981; Wickens and Stephenson, 1984; Zarkower *et al.*, 1986). In contrast, most yeast genes do not appear to use this AAUAAA mRNA 3' end processing signal (Zaret and Sherman, 1982). Instead, a number of different sequences have been implicated in yeast transcription termination. Thus, a consensus termination signal sequence in yeast is still ambiguous. From a comparison of yeast genes Zaret and Sherman (1982) have proposed a consensus TAG...TA(T)GT...TTT (where the sequences between the blocks is variable) as the sequence that signals transcription termination at a short distance downstream. However, Bennetzen and Hall (1982b) showed that the termini of several yeast gene transcripts occurred at the sequence 5' TAAATAAA/G 3' while Henikoff *et al.* (1983) and Henikoff and Cohen (1984) have proposed both 5' CAAT/GCTTTG 3' and 5' TTTTTATA 3' as possible termination sites. Irniger *et al.* (1991) has shown that the TTTTATA site works in an orientation dependent manner whereas Zaret and Sherman's triparite sequence functions bidirectionally.

Deletion of termination sequences results in read-through transcription which may substantially reduce the stability of the mRNA and thus the amount of gene product. Zaret and Sherman (1982) found that *CYC1*-specific RNA in a strain containing a 38bp deletion in the 3' noncoding region of *CYC1* is substantially longer than that of wildtype cells. In addition the total level of *CYC1*-specific RNA in the strain is only 5 to 10 percent of that found in the wildtype cells.

No convincing signals specifying poly(A) addition have been identified (Sutton and Broach, 1985). Although, Zaret and Sherman (1982,1984) have proposed that in yeast the polyadenylation of mRNA is coupled to transcription termination. Consequently, it has not been ruled out that some of the suggested termination sequences are involved in RNA processing rather than transcriptional termination.

In yeast, polyA tails average about 50 nucleotides (Phillips *et al.*, 1979) and were suggested to stabilise mRNAs by preventing 3' to 5' exonuclease attack (Littauer and Soreq, 1982). Since then, Bernstein and coworkers have demonstrated that it is a polyA: polyA binding protein interaction, rather than the polyA tail alone that stabilises mRNA (Bernstein *et al.*, 1989).

For genes that have intervening sequences (introns) RNA splicing of the primary transcripts is essential to produce functional mRNA. Most yeast genes do not contain intervening sequences, hence, excision of an intron is not an obligatory step in mRNA synthesis in yeast.

The processes mentioned above represent potential control points for the production of mature mRNA, thus regulating genes post-transcriptionally.

2.4.2a Translation

The final stage in the expression of the genes occurs when the information encoded in the nucleotide sequence of the mRNA is translated into the corresponding amino acid sequence constituting the polypeptide. Translation can be broken up into 3 phases; Initiation, Elongation and Termination. Although regulation can occur at all 3 stages, in most cases the rate at which an mRNA is translated is generally at the level of initiation (reviewed by Brown, 1989b).

The precise mRNA sequences required for efficient translation have not been strictly defined. In general, the most proximal AUG codon at the 5' end of the molecule sets the reading frame and initiates translation. Re-initiation at subsequent AUG codons does not occur (Kozak, 1987; Cigan and Donahue, 1987). Translation is terminated at a UAA, UAG or UGA codon.

Regulation of translation may also be determined by the protein sequence itself. Bennetzen and Hall (1982b) demonstrated a marked degree of preferential codon usage in highly expressed *Saccharomyces* genes. That is, more than 96 percent of the amino acids are encoded almost exclusively throughout their sequence by a set of 25 codons corresponding to major tRNAs; codons recognised by minor tRNAs are avoided. To study the affect of codon bias on gene expression Hoekema *et al.* (1987) replaced major codons with synonymous minor codons of the *PGK* gene. Their results showed a gradual decrease in protein level with increased minor codons. For further information on codon usage and its role in the optimisation of translation refer to the review by deBoer and Kastelein (1986).

2.4.2b Signal Sequences

Some yeast proteins contain signals that are responsible for their proper localisation within the cell. Proteins destined for the cell surface or secretion are synthesised in the form of precursors containing an essentially hydrophobic 5' peptide signal which is removed by proteolytic cleavage after localisation or at the beginning of the secretion process.

Comparison of many known signal sequences has revealed that these peptides have a low degree of sequence conservation, although they do possess an overall design in common (von Heijne, 1985). Typically, their length is between 15 and 30 residues and they show three distinct regions; a positively charged N-terminal n-region, a hydrophobic h-region and a more polar C-terminal c-region (von Heijne, 1988; Briggs and Gierasch, 1986). The

n-region varies in length and nature of amino acids but always has a positive charge. The h-region possesses hydrophobic residues linked together in what is essentially a random order (Gierasch, 1989; Heslot and Gaillardin, 1992b). The c-region has a more specific pattern with respect to the cleavage site and obeys the "-3, -1" rule: the residues at -1 and -3 must be small and uncharged. In contrast, bulky and charged residues are usually present at position -2 (von Heijne, 1983, 1984; Perlman and Halvorson, 1983).

In terms of genetic manipulation, of particular interest are those sequences that direct proteins out of the cell. *Saccharomyces cerevisiae* naturally secretes very few proteins. Examples of proteins destined for the periplasmic space and/or cell wall include invertase and acid phosphatase (Arnold, 1972; Linnemans *et al.*, 1977; Perlman and Halvorsen, 1983; Smith *et al.*, 1985), whereas the sex pheromones and the yeast killer toxin, are secreted out of the cell (Kurjan and Herskowitz, 1982; Julius *et al.*, 1984; Brake *et al.*, 1985). The later sequences have been extensively characterised for use in the expression of foreign proteins within yeast (Bitter *et al.*, 1984; Brake *et al.*, 1984; Lancashire and Wilde, 1987; Ernst, 1988; Skipper *et al.*, 1985; Tokunga *et al.*, 1988; Cartwright, 1992).

Much of the fundamental research described above is directed towards understanding the means by which yeast promoters are controlled and regulated. In recent years this information as been applied to extend our knowledge of heterologous gene expression in laboratory yeasts. However, much work still needs to be done to translate this information garnered from laboratory yeast in laboratory conditions for industrial purposes.

2.5 INDUSTRIAL APPLICATION OF RECOMBINANT TECHNOLOGY

The application of recombinant DNA technology to industrial yeast strains is a relatively recent area of research but is rapidly becoming a means of modifying the genetic properties of these yeast strains. As a consequence, these techniques will be more widely applied in years to come. It is possible that the construction of new strains will be used to further expand our knowledge of yeast genetics and moreover to elucidate the basic biochemical and physiological properties of the industrial yeasts used.

Most recombinant work to date has been done with brewing yeast strains while comparatively little is known about the molecular biology of wine yeasts. Several yeast properties of importance to fermentation performance are controlled by one or two genes. As a consequence, research has concentrated on reducing process costs and improving aspects of fermentation efficiency. Initially, the primary concern is to alter these properties without changing flavour and aroma of the final product. Nevertheless, flavour and aroma

are also controlled by yeast genes (although difficult to define genetically) and ultimately these may also be targets for genetic engineering. The remainder of this Chapter will present examples of recombinant DNA technology advances with industrial strains.

2.5.1 Diacetyl

Diacetyl is an undesirable product, derived from acetolactate (an intermediate of valine biosynthesis), which diffuses out of yeast cells during alcoholic fermentations. Removal of diacetyl is a rate limiting step in alcoholic maturation and consequently may result in additional fermentation costs.

Attempts to reduce the diacetyl levels by both the beer and wine industries has resulted in the introduction or inactivation of a number of different genes associated with the production of valine and consequently, involved in diacetyl production. For example, diacetyl levels were reduced by gene replacement of inactivated copies of the *ILV2* gene (Gjermansen *et al.*, 1988) and by the introduction of functional *ILV5* gene copies (Gossens *et al.*, 1991) into brewing strains. Gossen *et al.* (1991) found that with low copy integration of *ILV5* diacetyl levels were reduced, although still slightly above the desired threshold value. Consequently, to increase the expression of *ILV5* this gene was placed under the control of the strong constitutive *GAPDH* promoter. However, the expression of the *ILV5* gene was found to be severely reduced under the control of this promoter as compared to the original *ILV5* promoter (Gossens *et al.*, 1993).

Alternatively, a bacterial α-acetolactate decarboxylase (*ALDC*) gene, which converts the diacetyl precursor α-acetolactate to acetoin was introduced into brewing yeast to reduce the diacetyl levels (Sone *et al.*, 1987, 1988; Suihko *et al.*, 1989; Fujii *et al.* 1990; Tada *et al.*, 1995). Fermentation tests showed that the transformants were able to reduce diacetyl concentration of the fermented wort without any effect on the other characteristics of the beer (Sone *et al.*, 1987).

2.5.2 Sedimentation and Flocculation

Some strains of *Saccharomyces* have a tendency to agglomerate and to form flocs which produce a heavy sediment on the bottom of the fermentation vessel. This phenomenon, which is known as flocculation has long been recognised as very important in the production of alcoholic beverages because it greatly assists in the removal of the yeast from a ferment. Lack of flocculent ability may necessitate longer settling times, centrifugation and/or the use of fining agents to assist in yeast removal, ultimately resulting in increased production costs.

In order to achieve maximum conversion of sugar into ethanol and carbon dioxide, it is essential for the yeast to remain suspended in the fermenting liquid and not to flocculate. At the same time, the ability of the yeast to flocculate when the fermentation has completed, or reached the desired stage, is an obvious advantage. In other words, the yeast should ideally flocculate only at the desired stage in a fermentation - a stage that may vary with different beverages. However, a problem with flocculation is its variability: the nature of interactions among flocculent yeast cells is poorly understood. It is suggested that flocculation interactions are mediated by a specific cell surface recognition mechanism, involving lectin-like binding of surface proteins to polysaccharides on adjacent cells (Taylor and Orton, 1978; Miki *et al.*, 1980, 1982a; Stratford and Brundish, 1990). Furthermore, the onset of flocculation appears to be regulated by a variety of genetic and physiological factors and is influenced by the growth conditions and/or constituents of the growth medium.

Genetic studies of yeast flocculation were first reported by Gilliland (1951) and Thorne (1951). Subsequently, the genetics of flocculation has progressively been studied and a number of chromosomal genes affecting flocculation have been reported (see review by Stratford, 1992). Of these, a dominant flocculation gene (*FLO1*) has been extensively analysed both genetically and biochemically (Johnston and Reeder, 1983; Miki *et al.*, 1982a/b; Teunissen *et al.*, 1983a/b; Watari *et al.*, 1989; Watari *et al.*, 1994a; Bidard *et al.*, 1995) and has been used in the genetic manipulation of industrial yeasts (Watari *et al.*, 1994b).

Watari *et al.* (1994b) introduced the *FLO1* gene by chromosomal integration under the control of the *ADH1* promoter into a non-flocculent brewers yeast. Flocculent behaviour of the recombinant yeast was observed. However, the fermentation rate of the recombinant strain was slower and the number of cells in suspension lower than for the control strain. This was probably due to sedimentation of the recombinant cells early in the fermentation. The recombinant strain produced no off flavours and the flavour profile of the finished beer was similar to that of the control beer. Watari *et al.* (1994b) concluded that to retain the desired fermentation velocity, appropriate promoter constructs that target flocculation to the later stage of beer fermentation are essential.

2.5.3 Extracellular Enzymes

In the production of alcoholic beverages it is often necessary to add exogenous enzymes to improve fermentation productivity. Therefore, it is possible that genetic engineering may be able to produce yeast strains with a variety of enzymic activities and as a result lower process costs.

In brewers wort up to 20 percent of the sugar is present as non-fermentable dextrin and is inaccessible to yeasts. The addition of amyloglucosidase, an enzyme which hydrolyse these non-fermentable carbohydrates to yield glucose, has been used to maximise the conversion of cereal carbohydrates to alcoholic fermentation and to produce low carbohydrate beer.

In recent years, the brewing industry has made considerable progress in the construction of amylolytic strains of yeast by the introduction of glucoamylase genes. Glucoamylases have been cloned from the filamentous fungi *Aspergillus niger* (Boel *et al.*, 1984; Yocum, 1986) *Aspergillus awaamori* (Nunberg *et al.*, 1984) and *Schwanniomyces occidentalis* (Lanchashire *et al.*, 1989) and successfully expressed in *S. cerevisiae* (Innis *et al.*, 1985; Yocum, 1986).

The yeast *Saccharomyces diastaticus* also produces an extracellular glucoamylase which is capable of hydrolysing starch. This enzyme is encoded by one or more of a series of genes called *DEX* or *STA* (reviewed by Pretorious and Lambrechts, 1991). Glucoamylase genes from *S. diastaticus* have been isolated and successfully expressed in *S. cerevisiae* (Meaden *et al.*, 1985; Meaden and Tubb, 1985; Vakeria and Box, 1996).

Vakeria and Box (1996) introduced the *STA2* (*DEX1*) gene, under the control of two different promoters *PGK1* and *GAPDH*, into *S. cerevisiae*. The nature of the promoter on the expression plasmid was shown to influence both growth rate and stability of plasmids under non-selective conditions. The amounts of glucoamylase produced from the *GAPDH* promoter was very low under fermentation conditions yet the strain produced sufficient glucoamylase without any adverse affect on plasmid stability, cell physiology, the fermentation or product quality. This is in marked contrast to observations of the strain expressing glucoamylase from the efficient *PGK1* promoter which showed noticeable effects on plasmid stability and growth. Vakeria and Box (1996) concluded that high expression levels are not always the way to achieve stable modified characteristics in brewing strains and that judicious choice of promoter and vector can significantly influence the behaviour of a strain.

The other carbohydrase activity which is of interest to the brewing industry is the activity of β-glucanases. β-glucan (a structural polysaccharide) is present in both grape must and brewing wort and has the potential to cause haze and filtration problems in the fermentation of beer and wine. In the brewing industry these problems are overcome with the aid of exogenous commercial enzymes added at the beginning of fermentation or during wort production (Aschengreen, 1987). Therefore, yeast which are capable of hydrolysing β-glucans afford obvious process benefits. To this end, yeast that secrete enzymes to degrade β-glucans have been constructed.

The first experiments involving the isolation of endo-β-glucanase genes from *Bacillus subtilis* (Hinchliffe and Box, 1985; Cantwell *et al.*, 1985, 1987; Lancashire and Wilde, 1987) and their expression in *S. cerevisiae* demonstrated a low level of glucanase activity when the genes were driven by their own natural promoters and secretory signals (Hinchliffe and Box, 1985). This activity was increased by replacing the natural promoter with the yeast promoter *ADH1* (Cantwell *et al.*, 1985). However, significant levels of extra-cellular β-glucanases were only produced by the use of a yeast promoter, as well as a yeast signal sequence. As a consequence, plasmid stability during fermentation under non-selective conditions proved to be both plasmid and strain dependent. Also, at least one clone showed a slower fermentation rate (Cantwell *et al.*, 1987; Lancashire and Wilde, 1987). Nevertheless, the most stable transformed strain was capable of hydrolysing barley β-glucans to reduce beer viscosity and enhance filtration rate.

It has been suggested that the *B. subtilis* enzyme is not ideal for β-glucan hydrolysis during fermentation due to its relatively low hydrolytic activity at beer pH (Hinchliffe, 1985). Consequently, glucanase genes which are capable of hydrolysing barley β-glucans in the acid conditions of a beer ferment have been isolated from a variety of other organisms. Fungal endo-β-glucanase genes have been isolated from *Trichoderma reesei* (Arsdell *et al.*, 1987; Penttila *et al.*, 1987a/b; Enari *et al.*, 1987) and subsequently transformed into *S. cerevisiae*. Pilot brewing of transformed yeast strains produced beer with improved filterability, decreased viscosity and lower final β-glucan levels. Alternatively, Jackson *et al.* (1986) and Thomsen *et al.* (1988) introduced a barley gene (1-3, 1-4 β-glucanase isoenzyme II) into laboratory and lager yeast strains and succeeded in secreting active enzyme by fusion to the signal peptide region of mouse alpha-amylase. At this stage, no information is available concerning the ability of these yeasts to degrade β-glucan during fermentation.

Attempts have also been made to produce genetically engineered brewing yeast which secrete proteases but, so far, without success (Young and Hosford, 1987; Sturley and Young, 1988).

2.5.4 Aroma Properties

One of the most important characteristics of wine quality is its aromatic fragrance which depends partly upon the concentration of monoterpenes present in the must. These compounds are generally found as free volatile forms or as glycosidically bound non-volatile precursors (Williams *et al.*, 1982; Gunata *et al.*, 1985). This last fraction is a potential source of flavours which generally remain odourless in the traditional winemaking process (Gunata *et al.*, 1986). Consequently, enzymatic hydrolysis of these

grape glycosides by the addition of exogenous enzymes to must has been proposed as a strategy to enhance wine flavour and aroma (Aryan *et al.*, 1987; Gunata *et al.*, 1990a/b; Shoseyov, 1990). Taking this one step further, yeast which are capable of hydrolysing these aroma precursors would be of enormous potential benefit to the wine industry. As a result, Perez-Gonzalez *et al.*, (1993) introduced and expressed a fungal β-(1,4)-endoglucanase (*Egl1*) gene in wine yeast. The endoglucanolytic yeast secretes the fungal enzyme which is capable of hydrolysing monoterpenyl glycosidic bonds in must producing a wine with increased fruity aroma. The exogenous protein was secreted using a fungal signal peptide. However, approximately 50 percent of the produced protein remained within the cell.

2.5.5 Killer Factor

Killer factor was first recognised by Bevan and Makower (1963). Killer yeasts secrete polypeptide toxins which kill sensitive strains of the same genus and, less frequently, strains of different genera (Philliskirk and Young, 1975; Tipper and Bostian, 1984). In *S. cerevisiae* production of the killer toxin and immunity to it are determined by a cytoplasmically inherited double stranded RNA plasmid (Bostian *et al.*, 1980a). Based on the properties of the toxin, *Saccharomyces* killer yeasts have been classified into three groups: K1, K2 and K3 (Naumov and Naumov, 1973; Young and Yagiu, 1978). General reviews of killer yeast have been compiled by Young (1987), as well as Sturley and Bostian (1989) whereas a review of killer yeast and its relationship to the winemaking process has been compiled by van Vuuren and Jacobs (1992) and Shimizu (1993).

Many studies have been conducted to assess the efficiency of killer toxin on sensitive yeast strains. However, reports have been contradictory on the expression of killer activity under fermentation conditions (Lafon-Lafourcade and Riberau-Gayon, 1984; Delteil and Aizac, 1988; Petering *et al.*, 1991c). It is possible that differences in either composition of medium, fermentation conditions or strain sensitivity may account for discrepancies in reports of killer toxin efficiency. Consequently, the oenological significance of killer yeast is largely speculative. Nevertheless, it is believed that such strains could be used to restrict the growth of undesirable wild strains of *S. cerevisiae* and other closely related *Saccharomyces* species during fermentation.

Boone *et al.* (1990a) integrated the yeast K1 killer toxin gene into a K2 wine yeast to generate stable K1/K2 double killer strains. The modified strain has a broad competitive advantage over sensitive and killer strains of *S. cerevisiae* while its industrial performances in laboratory test are comparable to those of the original strain.

2.5.6 Malolactic Fermentations

Malolactic fermentation (MLF), which can be carried out by several different species of lactic acid bacteria, is an important secondary fermentation in the production of some wines. MLF involves the decarboxylation of L-malate to L-lactate and carbon dioxide and results in a reduction in acidity, microbial stability following the fermentation, and changes in wine flavour caused by the products of the bacterial fermentation (Kunkee and Goswell, 1977).

Malolactic fermentation usually occurs after alcoholic fermentation. However, poor development of lactic acid bacteria in wine often leads to delayed MLF or even prevents the reaction. Consequently, the ability of yeast strains to perform both alcoholic and malolactic fermentations in winemaking is being investigated with the view to achieving better control of MLF in oenology. To this end, attempts have been made to transfer the information necessary for the MLF from lactic acid bacteria to yeast.

Original work involved the cloning of the malolactic enzyme from *Leuconostoc oenos* (Lautensach and Subden, 1984) and *Lactobacillus delbrueki* (Williams *et al.*, 1984) and their transfer into yeast strains. However, in both cases, the level of conversion of malate to lactate by the engineered yeast strain was insufficient to be of practical benefit. This lack of success was suggested to be due to problems with expression of the cloned genes, or to the limited ability of the yeast host to take up malate. More recently, the malolactic gene has been isolated from *Lactococcus lactis* and put under control of the *ADH1* promoter in a multicopy plasmid. *S. cerevisiae* expressing the malolactic enzyme produced significant amounts of L-lactate during fermentation (Ansanay *et al.*, 1993; Denayrolles *et al.*, 1995). However, results indicate that the sole limiting step for *S. cerevisiae* in achieving malolactic fermentation, is malate transport (Ansanay *et al.*, 1996).

2.5.7 Increasing the Value of Spent Yeast

Saccharomyces cerevisiae is becoming a host of choice for use in the pharmaceutical and medical field to produce non-yeast products. Since fermenting practice results in the production of large quantities of waste yeast biomass, interest has been stimulated in developing uses for waste yeast to enhance its intrinsic value. Currently, spent yeast from industrial processes are being used to produce yeast extracts and as an ingredient in foods and flavourings. One approach to improving the value of this commodity has concentrated attention on the concept of producing high value protein which can subsequently be extracted from the spent yeast.

A novel process for enhancing the potential value of spent brewers yeast has been described by Hinchcliffe *et al.* (1987). Hinchliffe and coworkers developed a genetic process for the controlled synthesis of the high value therapeutic protein, human serum albumin (HSA) in brewing yeast. This involves the regulated expression of the HSA gene such that no HSA is produced during the course of the beer fermentation, but rather biomass is accumulated. Expression of the HSA gene is switched on in a post fermentation process.

Demand for heterologous protein production necessitates the development of expression systems capable of satisfying these needs. Growth phase must be separated from the production phase. Consequently, the use of late inducible promoters, which facilitate cell growth and hence biomass accumulation in the absence of heterologous gene expression, is central to the success of this new emerging area of fermentation technology .

2.6 CONCLUSION

The proceeding section has demonstrated the use of genetic engineering for improving industrial strains, although much of the work has highlighted the short comings in our knowledge of yeast physiology, genetic manipulation and the way they interact. Often a common problem is the expression of the introduced gene and the effect of the product on the host cell. In some cases, expression can be toxic or have negative effects on the characteristics of the transformed products, at least in certain stages of the fermentation cycle. Also, many common regulatable laboratory yeast promoters are often shown to be inapplicable for use in alcoholic fermentations.

However, with increasingly available technology and research it is slowly being recognised that the choice of promoter is not necessarily dictated by availability: a promoter can be isolated or designed especially to meet the economic and logistic factors involved in commercial production. Consequently, to obtain maximum productive expression conditions each situation needs to be evaluated independently and optimised for the environment and product. For the wine industry this concerns the development of modules for gene regulation that are compatible with the oenological process.

Thus, this thesis describes the isolation of sequences that are differentially expressed during anaerobic wine fermentations, the use of these sequences in the development of expression vectors and the application of this work to the production of genetically engineered wine yeasts for commercial purposes.

CHAPTER 3 : MATERIALS AND METHODS

3.1 CHEMICALS AND REAGENTS

All materials were of laboratory analytical grade and a list of solutions and media is given in Appendix A. The protocols of Sambrook *et al.* (1989) were used to prepare general stock solutions, bacterial media, buffers and reagents.

3.1.1 Enzymes

All restriction enzymes used during the course of this work were purchased from Promega or Boehringer Mannheim. Other enzymes were obtained from the following sources:

Calf intestinal phosphatase	:	Boehringer Mannheim
DNase 1	:	Promega
Klenow DNA polymerase	:	Boehringer Mannheim
Lysozyme	:	Sigma
Proteinase K	:	Sigma
Reverse transcriptase (M-MLV H$^-$ RT)	:	GIBCO BRL
Ribonuclease A (RNase A)	:	Boehringer Mannheim
T4 DNA ligase	:	Boehringer Mannheim
T4 DNA polymerase	:	Boehringer Mannheim

3.1.2 Radio-Chemicals

Radioactively labelled (α^{32}P) dCTP (1800Ci/mmol) and tritiated leucine (^{3}H) were obtained from Amersham.

3.2 STRAINS AND CLONING VECTORS

All bacterial strains and plasmids were obtained from general stocks within the Department of Plant Science, Waite Campus unless otherwise indicated. The wine yeast strain used in this study was obtained from the Australian Wine Research Institute.

3.2.1 Strains

Escherichia coli : The *E. coli* strain used for bacterial transformations was DH5α (F^-, *end*A1, *hsd*R17, *sup*E44, *thi*-1, λ-, *rec*A1, *gyr*A96, *rel*A1, D (*arg*F-*lac*ZYA), U196, *f*80*lac*ZDM15). The bacterial stains used to propagate lambda DNA clones were C600*Hfl* (*hsd*R^-, *hsd*M^+, *suo*E44, *lac*Y1, *ton*A21, *hfl*A150 (*chr*:Tn10)) and KW251 (F^-, *sup*E44, *gal*K2, *gal*T22, *met*B1, *hsd*R2, *mcr*B1, *arg*A81: Tn10, *rec*D1014) and were obtained from Promega.

Bacterial overnight cultures, for use as inocula in methods described later, were set up by the inoculation of a single bacterial colony into 2-5ml L-broth and grown shaking for 12 hours at 37^0C.

Saccharomyces cerevisiae : Australian Wine Research Institute Strain AWRI 796 (previously known as AWRI 3A) was obtained from Eschenbruch (1974). It was originally isolated in South Africa and is used for preparing both red and white wine. It is a low SO_2 and H_2S producer and has the K_2 killer property.

Yeast overnight cultures, for use as inocula in methods described later, were set up by the inoculation of a single yeast colony into 5ml YPD and grown shaking for 12 hours at 28^0C.

3.2.2 Cloning Vectors

λgt10 (*imm*434*b*527)	:	Promega
λgemm11	:	Promega
pAW220	:	Laboratory stock (Petering *et al.*, 1991a/b)
pTZ19U	:	Pharmacia

3.3 OLIGONUCLEOTIDES AND PCR PROGRAMS

PCR programs are set out in Appendix B. Synthetic DNA primers were determined using OLIGO 4.0 program on a Macintosh computer and were synthesised by Macromolecular Resources, Colorado State University. The synthetic oligo sequences are listed below;

ACT-F:	5' TCT GGC ATC ATA CCT TCT 3'	18mer
ACT-R:	5' TTC TCT TTC AGC AGT GGT 3'	18mer
ADH-F:	5' CCG CTC ACA TTC CTC AAG 3'	18mer
ADH-R:	5' TCT CTG GTG TCG GCT CTG 3'	18mer
λgt10-F:	5' GCA AGT TCA GCC TGG TTA AG 3'	20mer
λgt10-R:	5' TGA GTA TTT CTT CCA GGG TA 3'	20mer
M13-F	5' GTA AAA CGA CGG CCA G 3'	16mer
M13-R	5' CAG GAA ACA GCT ATG AC 3'	17mer
PHO-F:	5' GGC (C/T)CA (A/G)TA CGG (C/T)TA CAT 3'	18mer
PHO-R:	5' (A/T)GG (A/G)CC (A/G)TC (C/T)TG GTA GAA 3'	18mer
PGK-F:	5' TGA A(A/G)G TGA A(A/G)G CCA T(A/G)C 3'	18mer
PGK-R:	5' TGA ACG A(C/T)T GTG T(C/T)G G(C/T)C 3'	18mer
RP-F	5' ATT CGT ACC CAA CAA AAA 3'	18mer
RP-R	5' TAA GCG GTA CTA AAT TCG 3'	18mer
SMA-LINKER	5' CCC GGG TGC A 3'	10mer

3.4 MICROVINIFICATION TRIALS

3.4.1 Fermentation Procedure

Starter cultures were prepared by inoculating an aliquot of an overnight culture (3.2.1) of the desired yeast strain into YPD and incubating with vigorous aeration at 28^0C. Unless the starter culture is prepared under conditions of high aeration, low sugar to limit carbon catabolite repression, and high nitrogen, the culture will be deficient in lipid and carbohydrate reserves, resulting in a less than optimal fermentation. A serious deficiency will result in a stuck fermentation (Henschke, 1990).

After reaching stationary phase (24-36hrs), the cell density of the starter culture was determined by microscopic counts (3.4.2) and a sample taken to inoculate Sultana grape must (200-2000ml) to a density of 5×10^6 cells per ml. The must was supplemented with 1g/l DAP, 100mg/l myoinositol, 14mg/l nutriferm (Industrial Supplies) and 3mg/l thiamine-HCl. Equal amounts of glucose and fructose were also added to adjust the total fermentable sugar to 200g/l and the pH was adjusted to 3.5. The juice was sterilised by autoclaving for 10 minutes and sparged with nitrogen prior to inoculation.

The fermentations were carried out in fermentation flasks fitted with air locks. Juice aeration was controlled by nitrogen sparging of the juice prior to fermentation and sparging the head space during sampling. Samples were taken from the centre of the flask at regular intervals. These samples were removed anaerobically and aseptically during fermentation by needle and syringe through ports covered with a rubber septa. The fermentations were carried out at 18^0C with continuous gentle agitation.

Yeast cells were isolated for RNA extractions (3.5.2) and the fermentation progress was analysed by refractometer readings and ethanol concentrations (3.4.3). Yeast growth was measured by haemocytometer microscopic counts (3.4.2).

3.4.2 Microscopic Counts

Depending on the stage of yeast growth appropriate dilutions were made of the collected sample in isotonic saline to obtain countable numbers. Vortexing took place before diluting, between dilutions (if a serial dilution was required) and prior to counting. A small sample of the yeast cells were then transferred to a haemocytometer slide and the number of cells and percent budding cells were recorded. A haemocytometer slide has 2 counting areas both of which were used to obtain cell counts and then the two numbers averaged. To express the final cell number as cells/ml, cell counts were multiplied by 10^4

(haemocytometer slide factor) and the dilution factor used to establish the number of cells. In the last yeast cell count of the ferment methylene blue was present in the final diluent to distinguish between viable and non-viable cells. Viable cells appear colourless whereas non-viable cells appear or have concentrated areas of blue. Only viable cells were scored.

3.4.3 Wine Analysis

Sugar utilisation, as change in specific gravity (^{0}Brix), was determined by refractometer readings. The alcohol content was determined by near infra-red reflectance spectroscopy according to "Instructions for the Use of the Technicon 260 Infra-analyser" Bran and Luebbe, Australia. The instrument was calibrated according to Sneyd *et al.* (1990).

3.5 YEAST NUCLEIC ACID ISOLATION

3.5.1 Yeast DNA Isolation

Total yeast DNA was isolated by the following procedure. A yeast overnight culture (3.2.1) was grown in 10 ml YPD. Cells were pelleted by centrifugation at 3,000rpm for 5 minutes, washed once in zymolase buffer and resuspended in 500µl zymolase buffer containing zymolase to a total concentration of 1mg/ml. The suspension was then incubated shaking at 30^{0}C for 30 minutes. Spheroplasts were pelleted at 3,000rpm for 5 minutes, then resuspended in 100µl TE buffer. To this suspension 300µl of lysis buffer was added and the mixture incubated at 60^{0}C for 15 minutes. Following this incubation 200µl of 5M potassium acetate was added and the suspension incubated on ice for 30 minutes. After centrifugation at 10,000rpm for 20 minutes, the supernatant was carefully decanted into a fresh tube. To this supernatant an equal volume of absolute ethanol was added and the mixture was allowed to sit for 1 hour at room temperature.

The resultant DNA pellet, after centrifugation at 10,000rpm for 10 minutes, was vacuum dried and resuspended in 200µl TE buffer. The salt concentration of the aqueous phase was adjusted by the addition of 50µl of 5M potassium acetate followed by an incubation on ice for 30 minutes. After centrifugation at 10,000rpm for 20 minutes, the supernatant was carefully decanted again into a fresh tube. To this supernatant an equal volume of absolute ethanol was added and the mixture was left for 10 minutes at -80^{0}C. Centrifugation at 10,000rpm resulted in the pelleting of the DNA which was resuspended in a fixed volume of R40. The concentration of the DNA was determined by dilution of the sample before spectrophotometry for an absorbance value at 260nm. The relationship of one A_{260} unit to 50µg/µl DNA was used in the calculation of DNA concentrations (Sambrook *et al.*, 1989).

Total yeast DNA was used in the construction of a genomic library (3.7.2), amplification of yeast sequences by PCR (3.12.3) and in the electrophoresis (3.8.2) and transfer of DNA to nylon membranes (3.14.1).

2.5.2 Yeast Total RNA Isolation

Total RNA was isolated by a procedure based on that by Schmitt *et al.* (1990). The yeast culture was grown as per the fermentation protocol (3.4.1) and 10ml samples were taken at various stages of cell growth. The cells were harvested by centrifugation at 3,000rpm for 5 minutes and resuspended in 400µl AE buffer . After transfer to an Eppendorf tube 40µl of 10% SDS was added and the tube vortexed to mix. An equal volume of phenol (equilibrated with AE buffer) was added, the tube vortexed again and then incubated at 65^{0}C for 4 minutes. The tube was then rapidly chilled in liquid nitrogen until phenol crystals appeared and centrifuged for 2 minutes at maximum speed in an Eppendorf centrifuge. The upper aqueous layer was transferred to a fresh tube to which an equal volume of phenol/chloroform was added and left at room temperature for 5 minutes. To the extracted upper aqueous phase 40µl 3M sodium acetate pH 5.3 and $2^1/_2$ volumes of ethanol were added. Tubes were left overnight at -20^{0}C before the RNA was pelleted by centrifugation. The RNA pellet was washed once with 70% ethanol, dried and resuspended in a fixed volume of RNase free nanopure water.

Following RNA extractions, the absorbance values at 260nm and 280nm of each RNA sample was determined on a Shimadazu UV-Vis spectrophotometer UV-160A. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range of 1.8 to 2.2. The relationship of one A_{260} unit to 40µg/µl RNA was used in the calculation of RNA concentrations (Sambrook *et al.*, 1989).

Total yeast RNA was used for the isolation of polyadenylated RNA (3.5.3) and in the electrophoresis (3.8.3) and transfer of RNA to nylon filters (3.14.2).

3.5.3 Yeast mRNA Isolation

Polyadenylated mRNA was purified from the total RNA using the Promega "PolyA Tract mRNA Isolation System III" following the protocol set out by the manufacturer. Briefly, the system uses a biotinylated oligo(dT) primer to hybridise the 3' poly A region of mRNA. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA is then eluted by the addition of ribonuclease free water.

Polyadenylated RNA was used in the synthesis and construction of complementary DNA (3.7.1), radio-labelled first strand DNA (3.15.2) and *in vitro* translation studies (3.6.1).

3.6 PROTEIN SYNTHESIS

3.6.1 *In Vitro* Translation

A "Cell Free Wheat Germ Extract Protein Synthesising System" from Promega was used for the translation of isolated mRNA. Tritiated leucine was incorporated into newly synthesised protein following the manufacturers instructions. A TCA protein precipitation assay was done to determine incorporation of the labelled amino acid and the translation products were visualised by polyacrylamide gel electrophoresis and fluorography (3.6.2).

3.6.2 Electrophoresis of Proteins

Protein profiles were observed by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide separating gels (Appendix A) according to the directions the Bio-Rad "Mini Protean II" dual slab cell system. The gel was run in 1 x polyacrylamide electrode buffer at 200 volts for 60 minutes. Coomassie blue staining of the gel allowed visualisation of the proteins of the wheat germ cell extract to verify that the gel had run properly. Fluorography of the gel was achieved by soaking the gel in glacial acetic acid for 5 minutes, and then in a solution of 20% PPO in glacial acetic acid for 1 hour. The gel was rinsed under running water for 1 hour and dried under vacuum. Autoradiography was performed by exposing the dried gel to X-ray film (Fuji RX) at -80^{0}C for 4 to 8 days.

3.7 CONSTRUCTION OF LIBRARIES

3.7.1 cDNA Strand Synthesis

Double stranded cDNA with linked EcoRI/NotI adaptors was prepared using a "cDNA Synthesis Kit" purchased from Promega following the protocol set out by the manufacturer. Briefly, reverse transcription of the first cDNA strand was primed with a polydT oligonucleotide. After generation of the second strand, the double stranded cDNA was cloned by blunt end ligation to an EcoRI adaptor which also carried an internal Not I site. The linkered cDNA was ligated for 16 hours at 16^{0}C with EcoRI digested dephosphorylated λgt10 arms. The ligation mix was ethanol precipitated and washed prior to packaging and transfection (3.7.3).

3.7.2 Construction of a Yeast Genomic Library

Yeast DNA was partially digested with Sau3A and size fractionated by sucrose gradient centrifugation. The 10-30% sucrose gradient was centrifuged at 150,000g for 16 hours. Aliquots of 500µl were fractionated from the gradient and an aliquot of each fraction was analysed on a 0.7% agarose gel (3.8.2). Sucrose gradient fractions containing DNA fragments in the size range 15-20kb were pooled and dialysed with TE buffer. The DNA was then ligated to BamHI digested, dephosphorylated λgemm11 arms (Promega) in a 10µl ligation mix containing PEG at 13.6%, as per instructions of the manufacturer. The recombinant phage were precipitated in 0.6M NaCl, resuspended in TE and packaged using the Promega "Pack-a-Gene" kit exactly as described by the manufacturer (3.7.3).

3.7.3 Packaging and Transfection

Packaging extracts were obtained from Promega. Packaging of both the cDNA and genomic DNA ligation mixes into bacteriophage lambda particles was carried out according to the manufacturers instructions. Packaged cDNA was transfected into *E. coli* strain C600*Hfl* while packaged genomic DNA was transfected into KW251. The method was the same for both.

The encapsidated DNA was titrated by serial dilutions in SM buffer prior to plating. A starter culture was prepared by inoculating L-broth (50ml) containing 500µl 1M $MgSO_4$ and 500µl 20% maltose solution with 500µl of an overnight culture (3.2.1) of the desired *E. coli* strain and incubated shaking at 37°C until an OD_{600} of 0.6 was reached. 300µl of this culture was then incubated for 30 minutes at 37°C with 100µl of the appropriate encapsidated DNA dilution. This was then added to 10ml molten TB top agarose and poured immediately onto 15cm L-agar plates. Once the overlay was set, plates were inverted and incubated at 37°C for 12 hours.

3.7.4 Screening of Libraries

Libraries were screened using a variety of methods as described in this chapter. Filters of libraries were prepared as described in Section 3.14.3. Probing of cDNA libraries used radioactively labelled reverse transcribed RNA (3.15.2) for differential hybridisation. Genomic libraries were hybridised with probes prepared using the random primer method (3.15.1). Individual plaques were isolated and master plates of plaques made for reprobing, using the methods outlined below.

3.7.5 Preparation of Individual Lambda Lysates

Individual recombinant phages were isolated from agar plates by removing a plug of agar containing the desired plaque with a sterile borosilicate Pasteur pipette. This was then placed in 1ml SM buffer with a drop of chloroform and stored at 4^0C. Once left for 24 hours, it is ready for use.

3.7.6 Production of Master Plates

Once individual lambda lysates were purified, the phage were plated out in a master grid fashion for cross-hybridisation studies between clones. A bacterial lawn on a 15cm L-agar plate was obtained by placing 300µl of a bacterial culture (grown as described in Section 3.7.3) in 10ml TB top agarose and pouring this over the plate. Once set, 1-2µl of individual lambda lysates were carefully dotted in a grid pattern on the overlay. Plates were then inverted and incubated at 37^0C overnight.

3.8 RESTRICTION DIGESTIONS AND GEL ELECTROPHORESIS

3.8.1 Restriction Enzyme Digestions

All restriction enzymes were obtained from either Promega or Boehringer Mannheim. Restriction digests were performed in buffers supplied, and under conditions set by the manufacturer. Sufficient restriction enzyme was added to completely digest the DNA in 2 hours.

Partial restriction digests were carried out in buffer conditions recommended by the enzyme supplier. The enzymes were diluted using the recommended buffer to an activity of 0.1 unit/ml.

3.8.2 Electrophoresis of DNA

DNA fragment separations were carried out by submerged, horizontal gel electrophoresis. One part of DNA gel-loading buffer was added to five parts DNA sample prior to loading. DNA samples were electrophoresed in 0.7-3.0% agarose gels immersed in TAE buffer. After electrophoresis, gels were stained with ethidium bromide visualised on a 254nm UV light box. After being photographed, if necessary gels were prepared for transfer to nylon membranes (3.14.1) or the isolation of specific DNA bands (3.9.1).

3.8.3 Electrophoresis of RNA

Equipment was cleaned with 0.25N HCl to remove RNases before use. Total RNA was run on a 1.5% agarose denaturing RNA gel (Appendix A). Gels were poured and left at room temperature for 1 hour then pre-run in 1 x MOPS/EDTA buffer at 60 volts for 30 minutes.

Each RNA sample (5µg) was dried under vacuum and resuspended in 4.5µl RNA Buffer A and 9.5µl formamide/formaldehyde. The sample was incubated at 70^{0}C for 10 minutes and then chilled on ice for 2 minutes. RNA loading buffer (3µl) was then added and mixed well before loading into the gel. Samples were run at 60 volts for 30 minutes and then 100 volts for a further 1-2 hours. Gels were briefly stained with ethidium bromide and photographed under UV light before being prepared for transfer to nylon membranes (3.14.2).

3.9 CLONING OF DNA FRAGMENTS INTO PLASMID VECTORS

3.9.1 Isolation of DNA from Agarose Gels

DNA to be sub-cloned was digested with the appropriate restriction enzyme (3.8.1) and separated by gel electrophoresis (3.8.2). Fragments were recovered from the gel using the GENE CLEAN (BIO 101) kit following the supplier's instructions as outlined below.

The agarose containing the required fragment was placed in an eppendorf tube, 2 to 3 volumes of 6M sodium iodide was added and the agarose was dissolved by incubating at 50^{0}C for 5 minutes. A 5µl aliquot of Glassmilk solution (containing a silica matrix suspension) was added and incubated on ice for at least 5 minutes to allow the DNA to bind. The DNA/Glassmilk complex was pelleted by centrifugation for 5 seconds and the supernatant discarded. The pellet was washed 3 times with 500µl of a wash buffer containing NaCl and ethanol to remove all traces of sodium iodide.

The final pellet was resuspended in 5-10µl of water and incubated at 50^{0}C for 3 minutes. After centrifugation for 30 seconds, the supernatant containing the DNA was transferred to another tube and the elution step was repeated with another 5-10µl. The two eluants were pooled and centrifuged for 20 seconds to remove any traces of Glassmilk.

3.9.2 Preparation of Plasmid Vectors

For sub-cloning of library inserts and isolation of clones for sequencing vector pTZ19U was used. Vectors used for transformation of wine yeast (pAW220 and pAW222) are described in Chapter 7.

Vector DNA was linearised with the required restriction enzyme(s), extracted once with phenol/chloroform, ethanol precipitated and resuspended in 10µl of water. To prevent self ligation of the vector, linearised sequences were dephosphorylated prior to cloning, using Boehringer Mannheim calf-intestinal alkaline phosphatase as per the directions provided by the supplier. For sticky ends, the reaction was incubated for 30 minutes at 37^{0}C, while for blunt ends, a 15 minute incubation at 37^{0}C was followed by the addition of fresh enzyme and incubation at 55^{0}C for a further 30 minutes. In both cases the reaction was extracted twice with phenol/chloroform/isoamylalcohol and the DNA was precipitated with ethanol. The DNA was recovered by centrifugation and resuspended in TE buffer to a final concentration of 25µg/ml.

3.9.3 Ligation into Plasmid Vectors

DNA ligations were carried out using T4 DNA ligase under conditions specified by the supplier. 25-50ng of purified vector DNA was combined with a 1 to 3 fold molar excess of purified digested insert DNA in a 10µl reaction volume. Ligation reactions were left at room temperature for 2 hours or overnight at 16^{0}C. When the DNA fragment had incompatible protruding 5' and 3' termini it was treated in an end-fill reaction with the Klenow fragment of DNA polymerase I as per instructions of the manufacturer. This was carried out prior to purification of the fragment by gel electrophoresis (3.9.1).

3.10 TRANSFORMATION OF *ESCHERICHIA COLI*

Transformation of the *Escherichia coli* strain DH5α was performed according to the method of Hanahan (1983).

3.10.1 Preparation of Competent Cells

An overnight culture of *E. coli* strain DH5α was prepared as per Section 3.2.1. The following morning 0.5ml of this culture was placed in 50ml of SOB medium plus 0.5ml 2M Mg^{2+} and left to incubate, shaking at 37^{0}C until the OD_{600} ranged between 0.45 and 0.55. Cells were placed on ice for 10 minutes before being pelleted by centrifugation at

2,500rpm for 10 minutes at 4°C. The supernatant was removed, the cells gently resuspended in 8ml TFB buffer and then placed on ice for a 10 minute incubation. Cells were pelleted as before, the supernatant removed and resuspended in 2ml TFB buffer. To this, 70µl 1M DMSO was added and left on ice for 5 minutes, followed by 157µl 1M DTT and another 5 minute incubation. The cells were ready for transformation following the addition of 75µl 1M DMSO and a further 5 minute incubation on ice.

3.10.2 Transformation of Competent Cells

Competent cells (200µl) were added to the ligated plasmid DNA and placed on ice for 30 minutes. The transformation mix was then heat shocked at 42°C for 2 minutes and returned immediately to ice for 1 minute. After slowly warming to room temperature, 500µl of SOC was added and the cells incubated at 37°C for 45 minutes. Prior to plating the transformation mix was briefly centrifuged to pellet the cells. All but 200µl of the supernatant was removed into which the cells were resuspended before spreading directly onto an L-agar plate containing the appropriate antibiotic(s) and colour selection system. The plates were incubated at overnight 37°C.

Selection of all bacterial transformants was based on ampicillin antibiotic resistance (final concentration 50µg/ml). In the case of cloning into plasmid pTZ19U the presence of recombinant plasmids was also detected by a blue/white colony selection system, which involved plating on 25ml L-agar plates containing 50µl X-gal and 25µl IPTG.

Recombinant plasmid inserts were identified by small scale plasmid preparation isolation (3.11.1) and appropriate clones prepared for sequencing as per Section 3.11.3.

3.11 VECTOR DNA ISOLATION METHODS

3.11.1 Small Scale Plasmid DNA Isolation

Rapid DNA plasmid preparations were carried out in order to screen for recombinant plasmids from cloning experiments. 5ml of L-broth with the appropriate antibiotic was inoculated with a single bacterial colony and grown overnight at 37°C with aeration. The cells were harvested by centrifugation at 5,000rpm for 5 minutes and resuspended in 100µl Plasmid Solution 1. The suspension was left at room temperature for 5 minutes then transferred to ice with the addition of 200µl Plasmid Solution 2. Following a further 5 minute incubation 150µl 3M sodium acetate pH 4.8 was added and the tube inverted to mix. Chromosomal DNA and cellular debris were removed by centrifugation at top speed

in an Eppendorf centrifuge for 10 minutes. The supernatant was retained and extracted once with phenol/chloroform/isoamylalcohol. The upper aqueous layer was collected and precipitated by adding $2^1/_2$ times the volume of ethanol followed by an incubation of 5 minutes at room temperature. After centrifugation for 10 minutes the DNA pellet was washed once with 70% ethanol and resuspended in an appropriate amount of R40.

3.11.2 Large Scale Plasmid DNA Isolation

The preparation of large scale plasmid DNA was performed following the protocol of Sambrook *et al.* (1989) Following which, the plasmid DNA was purified by equilibrium centrifugation in a CsCl ethidium bromide gradient also according to Sambrook *et al.* (1989).

3.11.3 Preparation of Plasmid DNA for Sequencing

Preparation of DNA for sequencing was carried according to the Applied Biosystems "User Bulletin 18" for high quality template DNA for Taq cycle sequencing.

3.11.4 Lambda DNA Isolation

Small scale phage DNA isolation was by a modification of the method provided in the Promega "Protoclone λgt10 System" technical bulletin. The protocol for phage DNA isolation is the same for both genomic and cDNA phage clones.

Phage particles were grown to desired numbers by infection of an *E. coli* strain in liquid culture. An overnight culture of KW251 or C600*Hfl* (3.2.1) was inoculated into L-broth ($^1/_{10}$ volume) containing 0.2% maltose and 0.01M $MgSO_4$ and incubated shaking at 37°C until an OD_{600} of 0.6 was reached. 0.5ml of this culture was then incubated for 15 minutes with 100µl purified lambda lysate (3.7.5) at 37°C. Following this, 5ml of L-broth containing 2% maltose and 0.01M $MgSO_4$ was added to the mixture and it was left shaking at 37°C until lysis occurred.

The phage were collected by recovery of the supernatant after centrifugation in 15ml polypropylene centrifuge tubes for 10 minutes at 3,000rpm. To this supernatant RNase and DNase at a final concentration of 5µg/ml was added and left to incubate at 37°C for 30 minutes. An equal volume of 20% PEG/2M NaCl solution was then added and the mix was set on ice for 1 hour. The precipitated bacteriophage particles were recovered by centrifugation at 10,000g for 20 minutes at 4°C. The supernatant was removed by aspiration and the tube was inverted on a paper towel to allow the fluid to drain away.

The pelleted bacteriophage particles were resuspended in 700µl L-broth, gently vortexed and transferred to a 2ml Eppendorf tube. To this, 700µl DE52 was added and the tubes inverted 20-30 times. The tubes were spun at top speed in an Eppendorf centrifuge for 5 minutes and the supernatant transferred to a fresh tube. To the supernatant, 13µl proteinase K (0.1mg/ml) and 32µl 10% SDS were added and left to incubate at room temperature for 5 minutes. 130µl 3M potassium acetate was then added and the mixture heated to 88^{0}C for 20 minutes, followed by an incubation for 10 minutes on ice. Tubes were then spun at top speed in an Eppendorf centrifuge and the supernatant collected. DNA was precipitated by adding an equal volume of isopropanol to the supernatant and incubating at -20^{0}C for at least 1 hour. The DNA was pelleted by centrifugation and washed in 70% ethanol. After drying, the DNA pellet was resuspend in a fixed volume of R40.

Isolated phage DNA was digested with appropriate restriction enzymes (3.8.1) and run on DNA agarose gels (3.8.2) for visualisation of inserts. Inserts were extracted from gels (3.9.1) for subcloning (3.9) or for use as radioactively labelled probes (3.15.1).

3.12 DNA AMPLIFICATION BY POLYMERASE CHAIN REACTION

3.12.1 PCR Amplification of λgt10 Inserts

The bacteriophage vector λgt10 contains a unique EcoRI site within the phage 434 immunity region (*imm*434). Cloning in this site allows selection for recombinant phage. The use of primers, λgt10 forward and reverse, specific for and complementary to the *imm*434 regions on either side of this cloning site, enabled reliable PCR amplification of inserts up to 3 kilo base pairs.

A phage plaque was cored out using a sterile Pasteur pipette and transferred into a 1.5ml Eppendorf tube containing 100µl of SM buffer. The tube was left at 4^{0}C for at least 2 hours to allow diffusion of the phage particles into the buffer. A 10µl aliquot of the phage sample was then transferred to a small PCR tube and boiled for 10 minutes to denature the phage protein coat and the DNA. The boiled sample was chilled on ice and spun down before the addition of the PCR reaction mix (Appendix A) to a final volume of 25µl. The reactions were carried out in an MJ-Research thermal cycler using PCR program U55 (Appendix B). Products were visualised by gel electrophoresis (3.8.2) and if the correct insert size was obtained, the DNA was used directly from the PCR tube for radio-labelling (3.15.1).

3.12.2 PCR Amplification of pTZ19U Inserts.

DNA sequences cloned into plasmid vector pTZ19U containing M13 forward and reverse primer sequences were amplified using PCR and used as DNA probes during hybridisation studies.

50ng of plasmid DNA was placed in a PCR tube with PCR reaction mix (Appendix A) to a final volume of 25µl and cycled in a MJ-Research thermal cycler using PCR program U55 (Appendix B). Products were visualised by gel electrophoresis (3.8.2) and if the correct insert size was obtained DNA was used directly from the PCR tube.

3.12.3 PCR Amplification of Yeast Sequences

Synthetic DNA primers for amplification of yeast sequences were determined as described in Section 3.3. Listed below are the names given to the synthesised oligos and the yeast sequences they amplified;

RP-F and RP-R:	L45 ribosomal protein promoter
ACT-F and ACT-R:	Actin gene
PGK-F and PGK-R:	*PGK1* gene
ADH-F and ADH-R:	*ADH1* gene
PHO-F and PHO-R	*PHO5* gene

The ribosomal protein promoter sequence was subsequently used in a cloning experiment (3.9) whereas the other amplified yeast sequences were all used as radioactive probes (3.15.1).

Yeast DNA was diluted and 0.1µg in a total of 10µl placed in a PCR tube. To this, PCR reaction mix, with the appropriate primers, was added to a total of 25µl and the tubes placed in a MJ-Research thermal cycler PCR machine. Tubes containing primers ACT, ADH and RP were run through program U50 (Appendix B) and tubes with primers PHO and PGK used PCR program U45 (Appendix B).

3.13 SEQUENCING

DNA inserts to be sequenced were cloned into pTZ19U. Template preparations were prepared as described in Section 3.11.3. The DNA concentration of each plasmid preparation was determined by spectrophotometry as described in Section 3.5.1 and adjusted to 0.1μg/μl. The subsequent sequencing reactions were set up using the "Primer Ready Reaction Dye-Deoxy Terminator Cycle Sequencing Kit" according to the manufacturer. The reactions were cycled through PCR program SEQ55 (Appendix B) before being ethanol precipitated and briefly dried. Separation of the sequencing reaction products was carried out on a ABI automated DNA sequencer (Applied Biosystems). The sequence data was analysed using Sequence Editor (SEQ-ED; Applied Biosystems) run on a Macintosh computer. Open reading frames, amino acid sequence and restriction maps were determined using computer program DNA Strider.

3.13.1 Computer Sequence Analysis

Searches for similarities between deduced protein sequences and nucleic acid sequences was carried out with sequence databases such as Genbank, EMBL, PIR and Swiss-Prot. Data base searches were carried out using FASTA (Pearson and Lipman, 1988) and the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

3.14 PREPARATION OF HYBRIDISATION FILTERS

3.14.1 Transfer of DNA to Nylon Membranes

DNA fragments separated by agarose gel electrophoresis (3.8.2) were transferred to Hybond-N nylon membrane (Amersham) by capillary blotting (Southern, 1975). Gels were made ready for transfer by soaking in denaturing solution for 20 minutes. Upon completion of the transfer, membranes were briefly rinsed in 2 x SSC and the DNA fixed to the membrane by placing it in 0.4M NaOH for 20 minutes. Finally, the filter was soaked in neutralising solution for 5 minutes before being ready for hybridisation (13.6.1).

3.14.2 Transfer of RNA to Nylon Membranes

RNA was separated by denaturing electrophoresis (3.8.3) and transferred to membranes by capillary blotting (Southern, 1975). Gels were made ready for transfer by soaking in 20 x SSC for 30 minutes. RNA was transferred from the gel to Hybond N+ nylon membrane overnight by capillary blotting in 20 x SSC. After transfer, membranes were rinsed in 2 x SSC and UV cross-linked for 7 minutes before being baked at 40^0C for 1 hour.

3.14.3 Transfer of Plaques to Nylon Membranes

Phage were plated as described (3.7.3) using 13.5cm disposable Petri dishes at a concentration of approximately 1,000 plaques per plate or according to a master grid plan (3.7.6). A dry, circular, 13cm diameter Hybond N+ membrane filter was placed on the surface of the bacterial lawn containing the phage plaques and marked so as to permit unambiguous determination of the orientation of the membrane relative to the plate at a later time. The membrane was carefully removed and briefly air dried, colony side up, before being treated, in assembly line fashion, by exposure to the following solutions: denaturing solution - 5 minutes, neutralising solution - 7 minutes and 2 x SSC - 3 minutes. Exposure consisted of placing filters colony side up on Whatman 3 MM filter paper saturated with each respective solution. After air drying, the filter was baked at 80^{0}C for 30 minutes to fix the DNA then washed in stripping solution at 65^{0}C for 10 minutes to remove bacterial debris before being ready for probing.

3.15 PREPARATION OF RADIO-LABELLED PROBES

3.15.1 Radio-Labelling of DNA Samples

DNA inserts for use as probes were isolated from gels as described in Section 3.9.1 or directly amplified by PCR reaction (3.12).

Probes for hybridisation studies were prepared by random primer radio-labelling of double stranded DNA with (α^{32}P) dCTP. The DNA template (<1μg) plus 9mer random primers (0.1μg/ml) in a total of 8μl, were boiled for 5 minutes then chilled on ice for a further 5 minutes. This was then added to 12.5μl of 2x oligolabelling mix, 3μl of (α^{32}P) dCTP and 1.5μl of klenow enzyme (1 unit/μl). The sample was then incubated at 37^{0}C for 60 minutes. The radio-labelled DNA fragments were purified using a Sephadex G-100 column (3.15.3) and denatured by boiling for 5 minutes before addition to the hybridisation solution.

3.15.2 Synthesis of Radio-Labelled First Strand DNA

Single stranded complementary DNA probes, reverse transcribed from mRNA templates, were used in the differential hybridisation of the cDNA libraries.

To prepare cDNA probes, messenger RNA (1-5μg) was made up to a final volume of 10μl using RNase free H_20. To this 1μl oligo(dT) (500μg/ml) was added and the mixture incubated at 70^{0}C for 10 minutes before quickly chilling on ice. Radioactive first strand

reaction mixture (Appendix A) was prepared (8µl) and added to the RNA/oligo-nucleotide mixture. M-MLV H^- reverse transcriptase (200 units) was then added and the reaction mix incubated at 37°C for 60 minutes. The reaction was terminated by the addition of 7µl 1M NaOH and 7µl 1M HCl. The labelled first strand cDNA was purified using a Sephadex G-100 column (3.15.3).

3.15.3 Purification of Radio-Labelled DNA

A sterilised Pasteur pipette plugged with glass wool was loaded with Sephadex G-100 suspended in TE buffer and washed further with TE to produce a column. The labelling reaction was loaded onto the column and eluted with TE buffer. The eluent was monitored for radio-activity using a hand-held Geiger-Mueller counter and two peaks recorded for the elution curve. The first peak represented labelled DNA and the second unincorporated ($\alpha^{32}P$) dCTP.

3.16 HYBRIDISATIONS

3.16.1 DNA Hybridisations

Filters were briefly rinsed in 2 x SSC before being placed in a "Hybaid" hybridisation bottle and prehybridised in prehybridisation solution (10ml/filter) at 65°C for 2 hours to overnight. The prehybridisation solution was replaced with freshly prepared hybridisation solution (10ml/filter) plus denatured radioactive probe. The membranes were left to hybridise with the probe for 18 hours at 65°C with constant rotation. The filters were removed from the bottles and washed with agitation for 20 minutes at 65°C with each of the wash solutions (1-4) to remove background radioactivity. The membranes were then exposed to X-ray film between intensifying screens at -80°C. The exposure times were determined by the amount of detectable radioactivity on the membranes. Filters were made usable for reprobing by washing in boiling stripping solution for at least 20 minutes.

3.16.2 RNA Hybridisations

Filters were briefly rinsed in 2 x SSC before being placed in a "Hybaid" hybridisation bottle and prehybridised in RNA hybridisation solution (10ml/filter) at 42°C for at least 24 hours. After this time, the solution was replaced with freshly prepared hybridisation solution (10ml/filter) plus denatured radioactive probe. The membranes were left to hybridise with the probe for 24 to 48 hours at 42°C with constant rotation in a "Hybaid"

oven. The filters were removed from the bottles and washed with agitation for 20 minutes at 65^{0}C with each of the wash solutions (1-4) to remove background radioactivity. The membranes were then exposed to X-ray film at -80^{0}C. The exposure times were determined by the amount of detectable radioactivity on the membranes. Filters were made usable for reprobing by washing in boiling stripping solution for at least 20 minutes.

3.17 YEAST TRANSFORMATION METHOD

Alkali cation transformation of yeast was performed according to the method of Ito *et al.* (1983) with slight modification. Yeast cells were grown to late exponential phase at 28^{0}C in liquid YPD. Cells were harvested by centrifugation at 2,500rpm for 5 minutes, washed once in 10ml TE buffer, reharvested and suspended in 20ml LiOAc/TE buffer. Cells were left gently shaking at 30^{0}C for 1 hour. Approximately 8 x 10^7 cells were harvested and suspended in 100µl of LiOAc/TE buffer. Plasmid DNA (10µg) with 5µg salmon sperm carrier DNA was added in a total of 10µl to the cells and the suspension was incubated at 28^{0}C for 30 minutes. 700µl of PEG reagent was added and the mixture was vortexed before incubation at 28^{0}C for 1 hour. Cells were heat shocked by incubation at 42^{0}C for 5 minutes, harvested and resuspended in 1ml sterile water.

For the selection of transformants the cell suspension was spread on SD media containing 10µg/ml sulfometuron methyl.

3.18 β-GLUCURONIDASE (GUS) ASSAYS

Both GUS plate and cell suspension assays were compiled by an empirical investigation undertaken by Petering (1991a) to determine the appropriate conditions for the detection of GUS activity in transformed yeast strains. Each method involves inducing permeation of the yeast cell wall to facilitate the detection of GUS activity. The commercially available substrate used in these procedures is X-GLUC (5-bromo-4-chloro-3-indol β-glucuronide). β-glucuronidase cleaves the substrate X-GLUC to produce an indoxyl derivative which, upon oxidation, gives rise to an insoluble and highly coloured indigo dye. As a result, GUS activity can be detected by the presence of a blue precipitate.

3.18.1 GUS Plate Assay

Transformed wine yeast colonies were grown overnight on YPD media or SD media with 10μg/ml sulfometuron methyl both containing 50μg/ml X-GLUC. After approximately 36 hours growth, the plates were then overlayed with 0.8% agarose solution in 0.1M Na_2HPO_4 pH 7.0 and 1% sarkosyl. The sarkosyl present in the overlay induces leakiness to allow introduction of X-GLUC to the yeast cell wall/membrane. After 4 to 6 hours incubation at 37^{0}C a blue precipitate could be observed in colonies producing the β-glucuronidase enzyme.

3.18.2 GUS Cell Suspension Assay

Yeast cells were removed for an anaerobic wine ferment as described in Section 3.4.1 and for each tube their optical density checked to verify an approximate similarity between cell numbers. Cells were then pelleted and resuspended in 1ml 0.1M Na_2HPO_4 pH 7.0 plus 100μg/ml X-GLUC. Yeast cell extracts were then obtained by vortexing this suspension with glass beads (Sigma, 1000-1050 microns), to half the volume, for 20 minutes. Throughout the vortexing the cell suspensions were placed on ice to avoid overheating. After a brief incubation at 37^{0}C a blue precipitate is observed in suspensions if the β-glucuronidase enzyme is present.

CHAPTER 4 : YEAST GROWTH AND GENE EXPRESSION

4.1 INTRODUCTION

The composition of grape juice, the particular conditions of its fermentation (generally commencing aerobically and quickly becoming anaerobic), having a low pH value and a high sugar concentration, make wine yeast fermentations different to many yeast growth conditions. For this reason, information and technology from other food fermentations or from standard laboratory yeast studies are not necessarily directly applicable to oenology. In recent years, our knowledge of yeast physiology, metabolism and gene expression has advanced enormously, but use of this knowledge for alcoholic fermentations has been limited.

To understand the role of *Saccharomyces cerevisiae* yeast in fermentation it is essential to know: the kinetics of growth; the biochemical properties of the yeast; the chemical changes they produce; and the influence of fermentation factors upon both growth kinetics and metabolic change. This Chapter aims to examine mono culture *S. cerevisiae* wine fermentations by monitoring cell growth and the changing fermentation environment, in order to correlate this to the metabolic changes occurring within the cell, particularly in relation to gene expression. Of notable interest are those genes that are expressed at specific stages (non-constitutively) within the fermentation cycle.

4.2 YEAST GROWTH DYNAMICS AND FERMENTATION KINETICS

During fermentation, wine yeast use grape juice sugars and other constituents as substrates for their growth, converting these to ethanol, carbon dioxide and a variety of volatile (mostly in trace amounts) and non-volatile constituents, that contribute to the final chemical composition and sensory quality of the wine. Recent comprehensive reviews of wine yeast growth kinetics and fermentation profiles have been compiled by Reed and Nagodawithana (1991) and Fleet and Heard (1993).

In must, the growth cycle of *S. cerevisiae* wine yeast is representative of the classical phases of microbial growth; lag phase, exponential phase, stationary phase, and decline or death phases. However, with wine fermentation several variations in yeast growth may be noted due to low temperature, high concentrations of sugar, addition of large amounts of sulphur dioxide, and the lack of oxygen. These include: a particularly long duration;

limitation of total growth, corresponding to about 4 or 5 generations; the cessation of growth not arising from the exhaustion of sugar in the must; and a disproportion existing between the principal phases - the decline phase being able to last three or four times longer than the phase of multiplication (Lafon-Lafourcade and Ribereau-Gayon, 1984).

The kinetics of a fermentation are directly related to the yeast growth cycle. Upon transfer of a yeast culture to fresh must, there appears to be a short period in which cell activity is at a "low" lag phase and the inoculum of cells undergo a transition from a stationary to growth physiology. Eventually cells prepare to bud to produce daughter cells and the culture enters an "accelerated" lag phase. Yeast metabolic activity in this stage of growth is directed toward the production of biomass (Henick-Kling, 1988; Zoecklein *et al.*, 1990).

At the beginning of the exponential phase, the cells begin budding. These cells grow at a constant rate while they are dilute and have little influence on the medium. Sugar degradation is still mainly directed towards the production of cell biomass and only a small amount of ethanol is formed. Towards the end of the exponential phase, the number of budding yeast cells decreases and the growth of the yeast population slows. At this point, the limiting nutrients approach exhaustion and secreted inhibitory products accumulate, such that growth gradually declines through to a stationary phase. Stationary phase cells adapt to allow long term viability under nutrient limited conditions, but retain the ability to resume growth promptly if appropriate nutrients become available (Lillie and Pringle, 1980). The stationary phase in must usually begins when no more than half the sugars have been metabolised. Wine fermentations are normally completed by the metabolic activity of resting cells (Lafon-Lafourcade and Ribereau-Gayon, 1984), consequently the remaining sugars are slowly converted to alcohol during the stationary and decline phases (Ribereau-Gayon, 1985; Monk, 1986; Bely *et al.*, 1990).

The number of viable cells eventually begins to decrease at the end of stationary phase and decreases rapidly during the decline phase, at which time the yeast cell density also decreases (Lafon-Lafourcade, 1986). Finally, the metabolic activity of the yeast cell declines with age and the increasing concentration of alcohol (King and Beelman, 1986). In some situations the death phase coincides with the utilisation of the last of the available carbon source. However, initiation of the decline phase usually results from increasing membrane dysfunction. Membrane disruption arises from the combined effects of ethanol and carboxylic acid toxicity and a shortage in sterol precursors (Jackson, 1994). Further discussion on the toxic effects of ethanol on yeasts can be found in reviews by van Uden (1985, 1989), Casey and Ingledew (1986), Oliver (1987) and D'Amore *et al.* (1990).

The monitoring and enumeration of yeast and the changing fermentation parameters during the vinification process, provides valuable information about changes occurring during yeast fermentation. Consequently, to isolate sequences differentially expressed by *S. cerevisiae* wine yeast during anaerobic fermentations it was first necessary to characterise yeast cell growth and fermentation kinetics in laboratory scale fermentations.

4.2.1 Microvinification trials

Laboratory scale fermentations were prepared, with slight amendments, according to Henschke (1990) and as described in Chapter 3, Section 3.4.1. Fermentations were carried out under anaerobic conditions at 18^0C with gentle agitation. Samples were taken at regular intervals and assayed for yeast growth (cell count; Figure 4.1), percentage cell budding (Table 4.1) and the progress of fermentation was monitored by sugar concentration, as change in specific gravity, (refractive index; Figure 4.2), and ethanol concentration (% v/v; Figure 4.2). Mean values for each assay were plotted over time. The pH of the fermenting must was also checked at various intervals (Table 4.1). Sampling times covered the metabolically most active period of the fermentation: the first 100 hours.

The number of cells was established by haemocytometer cell count, at which time the percentage of budding cells was also recorded. Only budded cells of approximately equal size were recorded as 2 units. However, in the case of percent budding, all budding complexes were recorded. Cell size variation was observed during the yeast growth cycle: cells at the exponential growth phase ranged in size whereas cells at stationary phase were all small and compact.

The kinetic characteristics of yeast growth can be described in terms of; duration of the lag phase, growth rate, maximum cell population formed, and the duration of stationary and decline phases (Fleet and Heard, 1993). The lag phase is in general caused either by the use of an inoculum in which the population is in stationary or death phase, or by transfer to a medium of different physio-chemical properties and is a reflection of the fact that cells must undergo considerable changes in biochemical composition before growth can commence (Stanier *et al.*, 1971). As can be observed in Figure 4.1 there was a period of 6 to 12 hours before growth commenced and cells entered fermentation mode. To obtain the high concentration of cells needed to inoculate the must above 10^6 cells/ml, it was necessary to grow cells to exhaustion, that is, to a stationary metabolic state. Furthermore, cells were not pre-adapted to juice. Therefore, after inoculation, adjustment by the cells to high sugar concentrations (osmostress response), as well as a shift from aerobic respiration to anaerobic fermentation, was required.

Figure 4.1 : Single culture *Saccharomyces* yeast growth curve (cell count) during an anaerobic fermentation of supplemented Sultana grape juice incubated with gentle agitation at 18^0C.

Figure 4.2 : Sugar utilisation (refractive index, ^{0}Brix) and ethanol concentration (% v/v) during an anaerobic fermentation of supplemented Sultana grape juice incubated with gentle agitation at 18^0C.

Table 4.1: Percentage of budding cells and pH values at various stages of the fermentation.

Figure 4.1

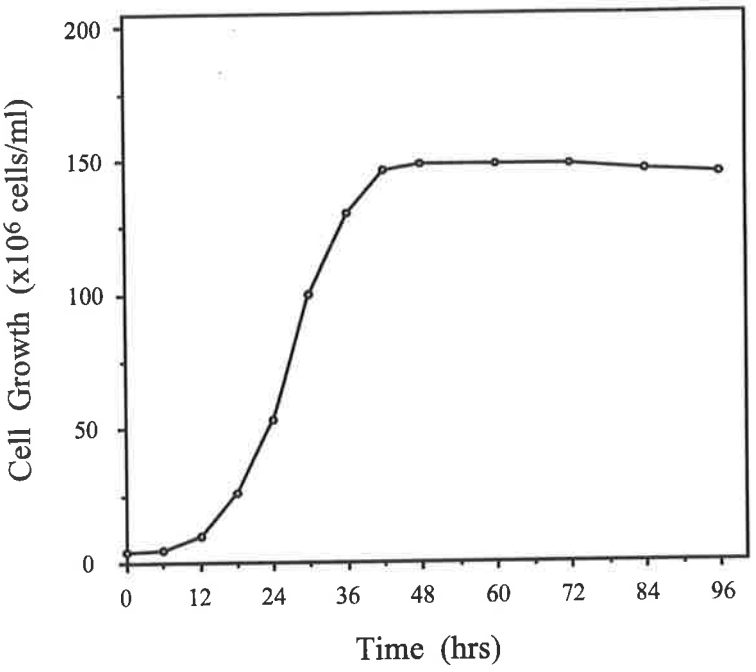


Figure 4.2

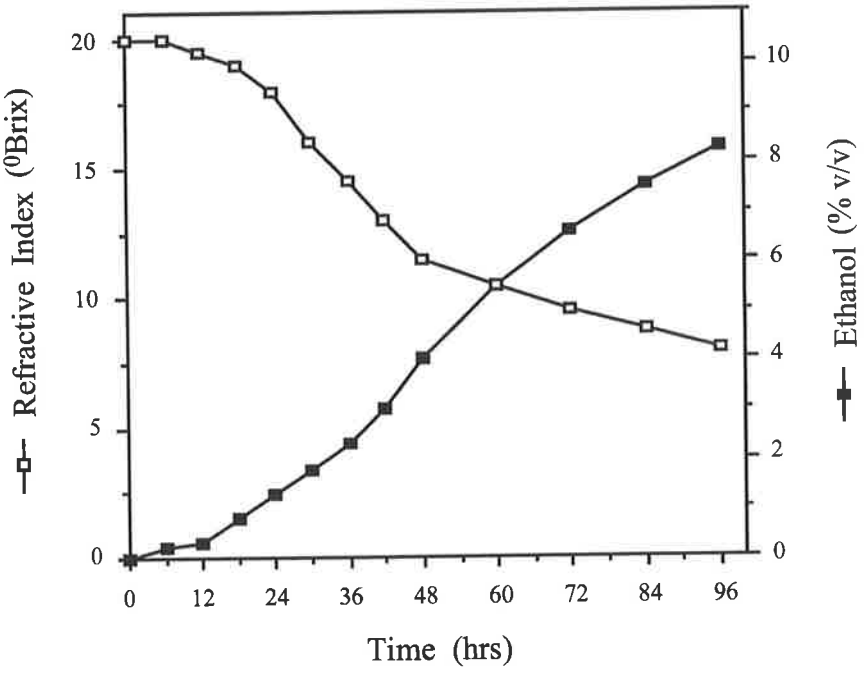


Table 4.1

Time (hrs)	0	24	42	72	96
% Budding	0	45	20	0.5	0
pH	3.5	3.4	3.2	3.3	3.2

Once fermentation began, the cell number increased quickly in the first 40 hours involving approximately 5 doublings, to reach a plateau of 1.5 x 10^8 cells/ml. All exponential phase cells examined were viable and many actively budding cells were observed (Table 4.1).

Stationary phase is generally characterised by a growth rate of zero after the culture has reached a maximum density. However, when a culture is grown in a complex medium and growth is limited by the accumulation of toxic products, constant cell numbers may be maintained for a time by the marginal growth of some members of the population being counterbalanced by the death of others (Stanier *et al.*, 1971). In this fermentation, a maximum density of 1.5 x 10^8 cells/ml was reached at about 40 hours and maintained until 72 hours. During this time a small percentage of cell budding was observed.

Cells were stained with methylene blue during the final cell count to identify non-viable cells. Only a small percentage of dead cells were detected, accounting for the slight decrease in cell number observed. This indicates that the cell decline phase had commenced.

Samples were also analysed for the progress of fermentation by refractometer readings to measure the sugar concentration as a change in specific gravity, and the alcohol content was determined by using near infra-red reflectance spectroscopy (Sneyd *et al.*, 1990) (Figure 4.2). The fermentable sugars of grape juice are glucose and fructose. The original must was supplemented with equimolar concentrations of glucose and fructose to raise the concentration of fermentable sugars to 200g/l at the commencement of fermentation. Nearing 100 hours into the ferment, between 65 and 75 percent of the total sugar had been utilised and converted to approximately 8 percent (v/v) ethanol. Upon completion of the fermentation the residual sugar concentrations for fructose and glucose should be 1 to 3g/l (Henick-Kling, 1988; Wibowo *et al.*, 1985) and the ethanol concentration may range from 10 to 15 percent. The final ethanol concentration may vary depending on such conditions as the yeast strain used, the original sugar concentration, pH and temperature (Reed and Nagodawithana, 1991).

4.3 GENE EXPRESSION

The changing physio-chemical conditions produced in the fermenting juice progressively modify yeast metabolism. This is reflected in the phases of growth noted above, the related adjustments in the nutrient and energy status of cells and the changing environment as compounds are absorbed and released throughout the fermentation. These major adaptive changes in cellular metabolism and physiology have been shown in many cases to

be co-ordinated by a complex set of regulatory processes that exert their influence at the level of gene transcription and, consequently, the final yield of proteins. Hence, differences in the metabolic activity of cells, at various stages of the fermentation growth cycle, can be visualised by the comparison of protein products.

Since the rate of synthesis of any protein is generally proportional to the cytoplasmic abundance of mRNA, an *in vitro* translation experiment of isolated mRNA can demonstrate the types of proteins being synthesised at the time of RNA extraction. If differences are observed in the protein profiles, these RNA transcripts may be used in the construction of cDNA libraries for the eventual isolation of those sequences that are non-constitutively expressed.

4.3.1 *In Vitro* Translation

The *in vitro* translation of yeast mRNAs extracted at regular intervals (every 12hrs for the first 72hrs) of the growth cycle during a fermentation, was performed using a Promega wheat germ system in the presence of tritiated leucine as described by the manufacturer. At the same time, *in vitro* translation of Brome Mosaic Virus RNA (provided in the kit) and a reaction devoid of mRNA served as positive and negative controls. Subsequent electrophoresis on SDS polyacrylamide gels, fixation, fluorography, drying and exposure to autoradiograph film allowed examination of the protein profiles at each extraction time (Figure 4.3). RNA extraction times T1 to T3 (12-36hrs) represent the lag and exponential phases, T4 to T6 (48-72hrs) represent the stationary and the beginning of the decline phase of the yeast growth cycle. Poor RNA stability during cell decline made analysis of gene expression after 72 hours difficult.

As SDS polyacrylamide gels separate proteins largely on the basis of molecular weight, each band can represent a number of structurally distinct protein species with the same electrophoretic mobility. However, immediate differences can be observed by visual comparison of individual protein profiles. Many bands are similar across all lanes but variations in the presence, absence or intensity of bands occurs with the change from exponential to stationary growth (36-60hrs) (Figure 4.3).

The expected pattern of gene expression during a wine fermentation is from a diverse set of genes during cell multiplication to a less diverse population upon cell entry into a resting state during the stationary phase. Boucherie (1985) has shown that the rate of protein synthesis in stationary phase yeast cells in minimal medium falls to 10 percent or less of that observed in exponentially growing cells. In this study, the complexity of bands

Figure 4.3

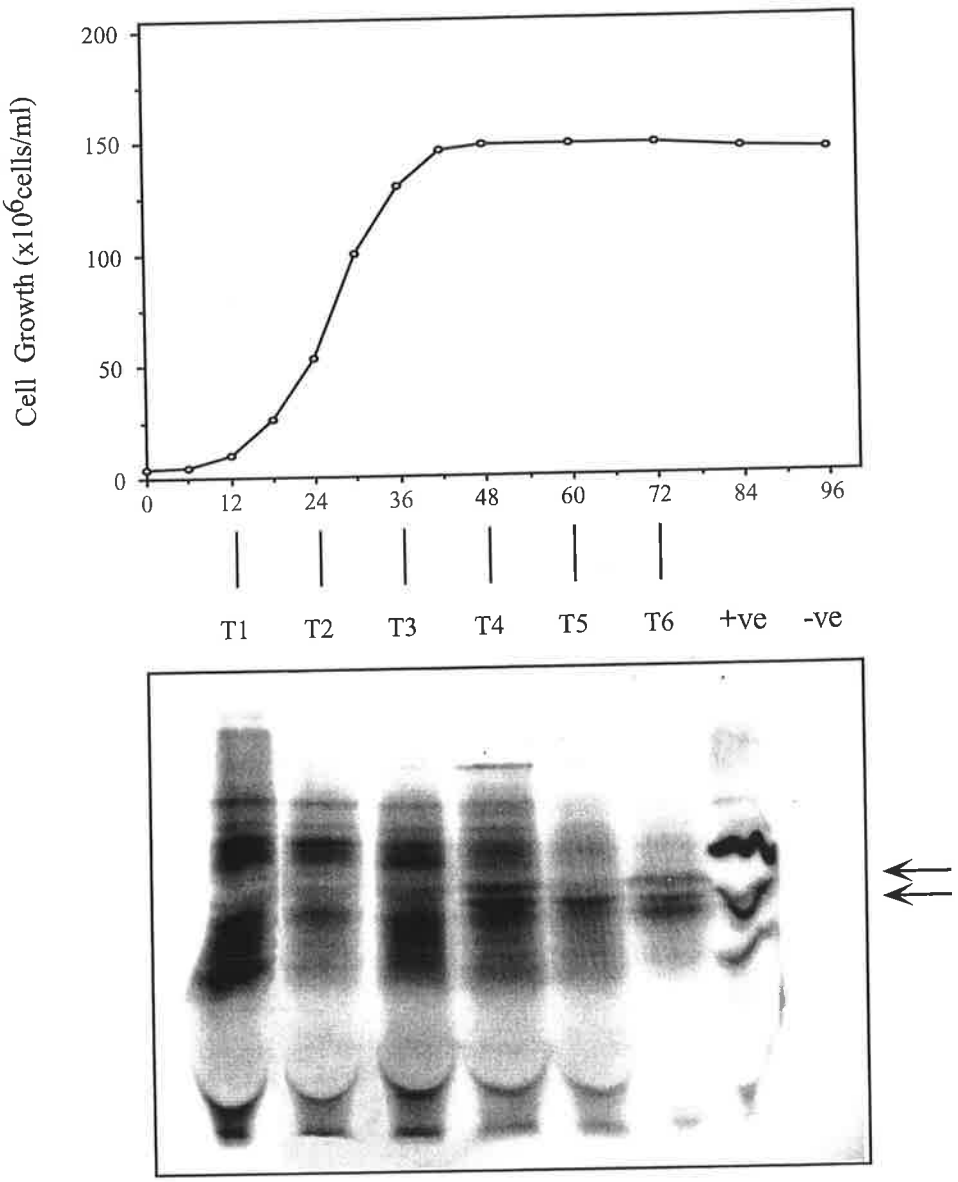


Figure 4.3 : *In vitro* translation of mRNA extracted from cells at different stages of growth during an anaerobic wine fermentation.

Extraction times ; T1 -12hrs, T2 -24hrs, T3 -36hrs, T4 -48hrs, T5 - 60hrs, T6 -72hrs.

Extraction times T1 to T3 represent the lag and exponential phases of yeast growth and T4 to T6 represent the stationary and decline phase of yeast growth.

Positive control - Brome Mosaic Virus RNA
Negative control - No RNA

appears to be greater during the first 48 hours and many bands are lost in the protein profiles from 60 hours (T5) onwards. These bands may represent proteins which are only expressed in the early stages of fermentation.

Distinct bands (indicated by arrows) are absent in the first 36 hours but arise at T4 (48hrs) and persist through to the end. In general, the intensity of a band provides a rough indication of the proportion of RNA it is represented by in the total mRNA population. The indicated bands appear to be increasing in intensity when compared to the weak signal given by the remaining bands at the final two extraction times. Thus, these bands appear to represent proteins which are selectively expressed in the later stages of fermentation.

The *in vitro* translation products of different stages of yeast growth indicate that a transcriptional response to the changing environment was occurring. The types of genes differentially expressed at both early and late stages of an anaerobic wine fermentation will be discussed in the next Chapter.

4.4 DIFFERENTIAL HYBRIDISATION OF EXPRESSION LIBRARIES

A widely used procedure for the of isolation of non-constitutively expressed sequences is via differential hybridisation of genomic or cDNA clones to labelled cDNA. Differential hybridisation screening was originally developed in yeast and used for isolation of the galactose inducible genes (St John and Davis, 1979). Differential representation of cloned cDNA sequences in different mRNA populations are indicated by quantitative differences in hybridisation of these clones to cDNA probes reverse transcribed from the mRNA populations in question. The selected cDNA clones are, in turn, used as homologous probes to screen a genomic library for their full length transcript and associated regulatory sequences.

The remainder of this Chapter describes the application of a differential cDNA hybridisation approach to isolate cDNA clones of mRNA expressed non-constitutively during wine fermentations.

4.4.1 Construction of cDNA Libraries

The products of the *in vitro* translation indicate that mRNA integrity was preserved during purification because protein bands are well defined, with no indication of smearing due to translation from shortened transcripts. Also, due to the large size and range of proteins it can be concluded that full-length cDNA clones could be generated from the mRNA stocks (Figure 4.3).

Two cDNA libraries of approximately 10,000 clones each were made from exponential (Early; T1-12hrs) and stationary (Late; T5-60hrs) growth phase mRNA populations. Both libraries were constructed, with the same mRNA stock used in the *in vitro* translation assays, as outlined in Chapter 3.

The obvious different *in vitro* translation profiles of exponential compared to stationary mRNA indicates that each library should include a proportion of transcripts showing stage-specific expression. Differential screening of these libraries was used to identify these clones.

4.4.2 Isolation of Non-Constitutively Expressed Sequences

Quadruplicate lifts of the exponential cDNA and stationary cDNA libraries were hybridised to cDNA probes reverse transcribed from RNA transcripts isolated at 12, 32, 60 and 72 hours of an anaerobic wine ferment. Visual screening of the first round of hybridisations revealed clones with expression patterns of low level constitutive, high level constitutive and identified some clones that are differentially expressed in either library. A number of clones were selected for second round purification and re-screening. The subsequent re-screening verified 144 clones specific for the exponential phase library ("early" stage-specific) and 137 specific for stationary phase ("late" stage-specific) (Table 4.2).

4.4.3 Characterisation of cDNA Libraries

Both "early" and "late" cDNA clone sets were characterised with respect to the frequency of the non-constitutively expressed transcripts within each expression library, the diversity of clones isolated, the size of these clonal families and the range of insert sizes (Table 4.2).

Phage DNA was prepared and inserts sized after restriction endonuclease digestion. All cDNA clones screened contained inserts. Hence the library count was considered to be the effective library size and all plaques were considered to represent a yeast transcript. The size of the cDNAs ranged from 1.8kb to 0.2kb for the exponential library and 1.3kb to 0.1kb for the stationary library. In general larger cDNAs were observed within the exponential library.

As an approximation, the mRNA sequences of interest will be represented in the cloned double stranded cDNAs in proportion to their abundance in the original preparation of mRNA. Therefore, expression levels may be assessed by cross-hybridisation between clones to assess the diversity of clones and determine the size of the clonal families.

Table 4.2

Library	Library size	No. of stage-specific clones	% stage-specific per libary	No. of diverse clones	% diversity among clones	size variation among clones
T1	1.35×10^4	144	1.1	45	31	0.2kb-1.8kb
T4	1.14×10^4	137	1.2	19	14	0.1kb-1.3kb

Table 4.2 : Caracterisation of cDNA libraries with respect to size, frequency of non-constitutively expressed sequences, variation among these sequences and insert size.

The isolated early stage-specific sequences contribute 1.1 percent of the exponential (T1) library. A diverse range of sequences was identified through cross-hybridisations; thirty percent of the sequences were characterised as representing different clonal families, although some cross-reactivity was observed (Table 4.2). In most cases, each clonal family consisted of an equivalent number of clones suggesting an overall similarity in expression levels of the sequences. In contrast, the "late" stage-specific sequences were more homogeneous, accounting for 1.2 percent of the stationary (T5) library with only 14 percent of sequences being different. Cross-hybridisation identified clonal families represented by a single clone, as well as families containing up to 30 percent of the sequences isolated (Table 4.2). Thus the clones appear to represent both rare and highly abundant transcripts.

These results are consistent with the expected protein expression patterns of yeast during a wine fermentation: a large diverse range during cell growth and a small homogeneous range upon nutrient depletion and increased ethanol concentration.

4.4.4 RNA Hybridisation Analysis

The non-constitutively expressed cDNAs were identified by their differential expression in wine fermentations. In order to determine more accurately the profile of induction for certain clones, RNA was harvested and expression monitored by RNA hybridisation analysis.

Total yeast RNA was prepared from cells extracted at regular intervals (every 12hrs for the first 72hrs) during a wine fermentation. RNA hybridisation filters were prepared and probed with radio-labelled cDNA clones, as described in Chapter 3. Each hybridisation was performed with probe excess, therefore the intensity of the autoradiographic signal is a measure of the concentration of the specific mRNA in the RNA population. Messenger RNA levels relative to the level of actin mRNA were also measured by RNA hybridisation analysis to correct for differences in RNA yield between samples.

RNA hybridisations were conducted using at least 10 different cDNA clones specific for each stage as probes. Two basic expression patterns with slight variations were observed (Figure 4.4). Clones from the T1 ("early" stage-specific) cDNA library are strongly expressed during exponential growth phase, but not during the stationary and cell decline phases (Figure 4.4a). T5 ("late" stage-specific) cDNA library clones are not expressed during growth phase but are strongly induced from early stationary phase with expression persisting throughout stationary phase (Figure 4.4b). Detection of expression during cell decline was made difficult by poor RNA stability.

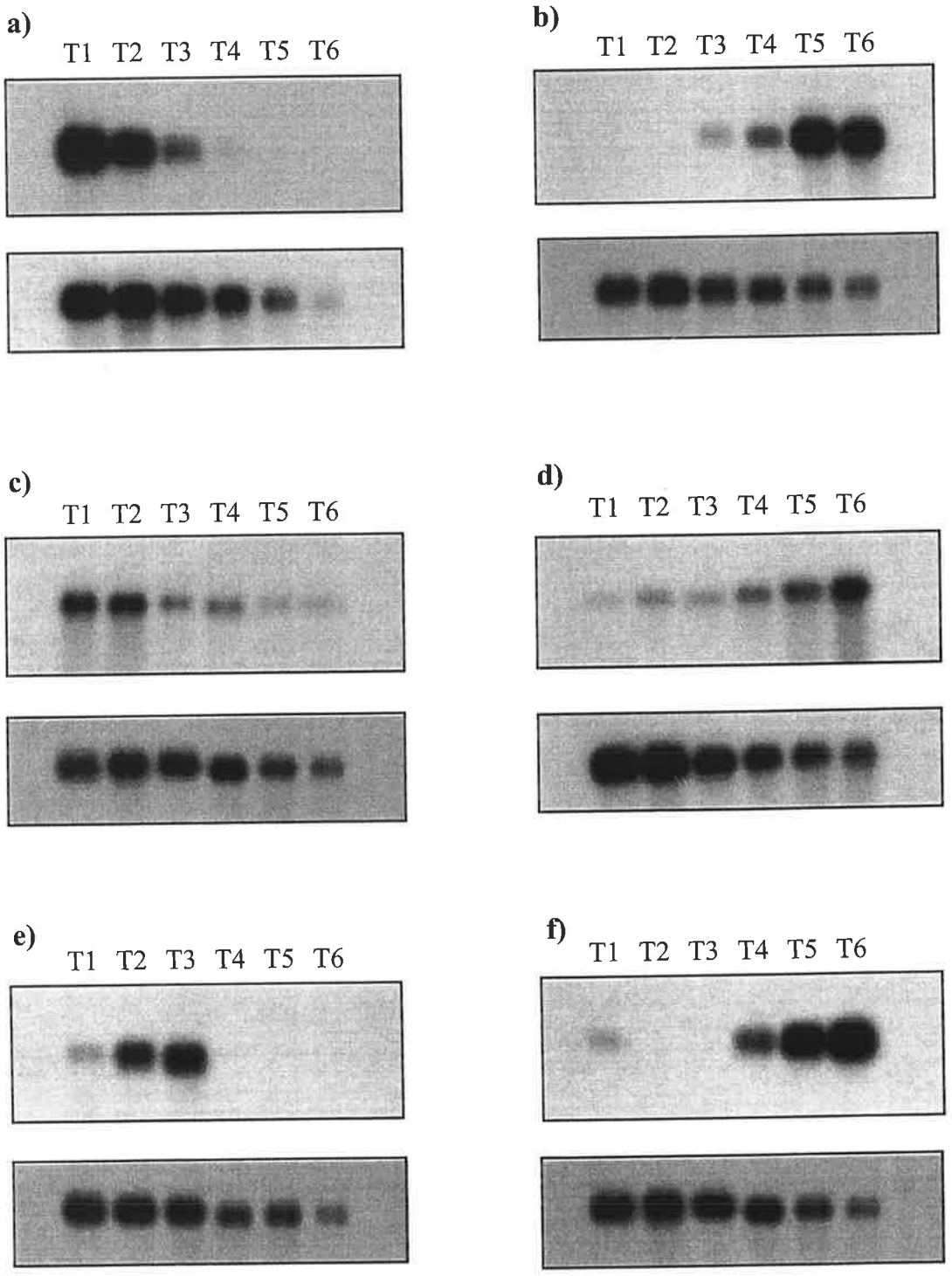
Figure 4.4 : Representative RNA hybridisation patterns of sequences isolated for their differential expression during anaerobic wine fermentations.

Filters contain RNA extracted, at 12 hourly intervals, from yeasts undergoing fermentation: T1 - 12hrs, T2 - 24hrs, T3 - 36hrs, T4 - 48hrs, T5 - 60hrs, T6 - 72hrs.

"Early" stage-specific patterns include a), b) and e) and "late" stage-specific patterns are represented by b), d) and f).

Actin control filters for each hybridisation pattern are also displayed.

Figure 4.4


a)
T1 T2 T3 T4 T5 T6
b)
T1 T2 T3 T4 T5 T6
c)
T1 T2 T3 T4 T5 T6
d)
T1 T2 T3 T4 T5 T6
e)
T1 T2 T3 T4 T5 T6
f)
T1 T2 T3 T4 T5 T6

One variation of these patterns consisted of basal expression before or after the induction of transcription (Figure 4.4c and 4.4d). Another variant, for "early" stage-specific clones, was low level expression occurring in the first 12 hours (lag phase) with strong expression persisting for the next 24 hours (exponential phase) and no expression for the last 36 hours (stationary phase) (Figure 4.4e). In another example, some "late" stage-specific clones, demonstrating the basic expression pattern stated above, also show a small amount of mRNA transcription within the first extraction time (Figure 4.4f).

4.5 DISCUSSION

To isolate sequences differentially expressed by *S. cerevisiae* wine yeast during an anaerobic fermentation it was first necessary to conduct laboratory scale fermentations, which model commercial fermentations, to establish yeast cell growth and fermentation kinetics. Figures 4.1 and 4.2 represent a typical fermentation profile which may be compared with those presented by others (Reed and Nagodawithana, 1991; Bisson, 1993; Fleet and Heard, 1993). Basic patterns are observed with slight variations arising due to composition of must, amount and preparation of the original inoculum, temperature, amount of agitation and available oxygen.

Initial experiments were conducted to look at the protein expression patterns during a ferment. Messenger RNA was extracted from specific stages of a ferment and translated *in vitro*. Subsequent electrophoresis of the *in vitro* translation products provided an indication that differential expression does occur due to the changing environment of the ferment and that this expression is tightly linked to the growth of yeast. Major differences were observed between cells in rapid growth and at stationary phase.

Alterations in the prevalence of several transcripts during the yeast life cycle and expression differences associated with the growth phases have been observed by others (Iida and Yahara, 1984a/b; Boucherie, 1985; Eglimez *et al.*, 1989; Bataille *et al.*, 1991; Ju and Warner, 1994; Destruelle *et al.*, 1994). Indeed, stationary phase cells have been shown to be physiologically, biochemically and morphologically distinct from exponentially growing cells (reviewed by Werner-Washburne *et al.*, 1993). Stationary phase cells are shown to be more resistant to killing by heat (Schenberg-Frascino and Moustacchi, 1972; Walton *et al.*, 1979; Plesset *et al.*, 1987) and cell wall degrading enzymes (Deutch and Parry, 1974; Piedra and Herrera, 1976; Zlotnik *et al.*, 1984; DeNobel *et al.*, 1990a/b), as well as having different RNase activity (Mills, 1972; Swida *et al.*, 1981), polyadenylate content (Sogin and Saunders, 1980; Boucherie, 1985; Choder, 1990), storage levels of carbohydrates (Lillie and Pringle, 1980), and a folded genome structure (Pinon, 1978; Pinon and Pratt, 1980) relative to exponentially growing cells.

Despite these physiological and biochemical changes, stationary phase cells contain half as much polyA mRNA as found in exponentially growing cells (Sogin and Saunders, 1980; Boucherie, 1985; Werner-Washburne *et al.*, 1989; Choder, 1991). This was confirmed in the *in vitro* translation experiment by the progressive decrease in the number of bands as the fermentation proceeds (Figure 4.3). However, not all transcripts decrease in abundance as cells enter stationary phase. Two-dimensional gel electrophoretic studies of radio-labelled proteins isolated from cells starved for particular nutrients suggest that many different proteins may be preferentially synthesised during entry into stationary phase (Iida and Yahara, 1984a/b; Boucherie, 1985; Destruelle *et al.*, 1994). A similar situation can be observed at the end of fermentation with the appearance of a number of strong bands in the *in vitro* translation gel (Figure 4.3). Some of the genes expressed may encode proteins for proteolysis, as well as many heat shock genes which are suggested to be involved in protein stabilisation and transport, necessary for stationary phase survival. For an extensive list of genes thought to be involved in entry and maintenance of the stationary phase, refer to the review by Werner-Washburne (1993).

Alternatively, genes expressed at the exponential stage of the yeast life cycle are commonly associated with growth, maintenance, division and general "housekeeping" of the cells. For example, ribosome biosynthesis, ubiquitous in living cells, has been shown to be directly proportional to the growth rate during the growth cycle of *S. cerevisiae* (Waldron and Lacroute, 1975; Warner, 1982; Warner *et al.*, 1985; Mager and Planta, 1991; Raue and Planta, 1991; Kraakman *et al.*, 1993; Ju and Warner, 1994).

Results of the *in vitro* translation also allowed an evaluation of the potential for differential hybridisation of cDNA libraries for isolating non-constitutively expressed wine yeast genes. Firstly, the products of the *in vitro* translation indicate that mRNA integrity was preserved during purification and, due to the large size and range of transcripts, it can be concluded that full-length cDNA clones could be generated. Secondly, as difference in the protein products were observed, this implies the resultant cDNA libraries made from the same mRNA population would contain unique sequences.

Research with laboratory yeast suggests that approximately 50 percent of the genome is transcribed under "normal" conditions (St. John and Davis, 1981a/b) with many genes always expressed at a basal level. Of the total RNA extracted from a yeast cell only 1 to 5 percent is due to transcription by RNA polymerase II (Struhl and Davis, 1981) and only a portion of these genes are non-constitutively expressed. Consequently, this necessitated the generation of large libraries to ensure the isolation of those sequences expressed at specific times. The differential cDNA hybridisation approach allowed screening of large

numbers of clones at a time and identified many non-constitutively expressed sequences. In practice, logistics of the screening process limited the approach to the detection of transcripts which appear at moderate to high levels in the RNA extractions used for construction of those libraries. Transcripts of very low abundance are best isolated from a cDNA library constructed from a mRNA population depleted of repeated sequences by screening with subtractive probes (Sambrook *et al.*, 1989). In addition, sensitivity of the hybridisation with heterogeneous cDNA probes was low so that RNA hybridisation analysis was required to more accurately assess patterns of expression of selected clones.

With RNA hybridisation analysis, differential expression was confirmed. Two obvious expression patterns were observed. "Early" stage-specific clones are strongly expressed during exponential growth phase, but not during the stationary and cell decline phases. "Late" stage-specific clones are expressed during the growth phase but are strongly induced from early stationary phase with expression persisting throughout stationary phase.

A similar expression pattern to that of the "early" stage-specific clone has been observed by Kraakman *et al.* (1993), as well as Ju and Warner (1994) in the level of mRNA for ribosomal proteins as a culture of *S. cerevisiae* passes through the growth cycle. They found that transcription of both ribosomal RNA and ribosomal protein genes disappears with decreased growth rate accompanied by a decline in total RNA content of nearly 50 percent and a decline in the number of ribosomes per cell to less than 25 percent of the maximum value.

As stated previously, some genes have been shown to be preferentially expressed at stationary phase and consequently show similar RNA hybridisation patterns as described above (Werner-Washburne, 1993). However, many "late" stage-specific genes were also seen to display a small amount of mRNA transcript within the first extraction time. One explanation for the presence of these transcripts within the lag phase of the growth cycle may result from stationary phase cells being used as the inoculum. However, this supposition requires a stability within the transcripts which has been shown to be decreased in stationary phase cells (Mills, 1972; Swida *et al.*, 1981). Another possibility remains that these transcripts belong to a set of stress response genes. As a result, low expression may be due to shock on the cells from high sugar concentrations (osmostress response), as well as the shift from highly aerobic to anaerobic conditions. A stress-inducible yeast gene *STI1* has been shown to have a similar expression pattern, with mRNA detectable in early exponential phase but decreasing below detection until cells enter stationary phase where *STI1* encoded mRNA accumulates again (Nicolet and Craig, 1989).

Considering the RNA expression patterns described above, it would be of interest to determine whether the differentially expressed genes isolated are casually associated with the growth cycle in yeast fermentations or are expressed as a result of the fermentation conditions. Furthermore, it may be of interest to determine whether the function of the gene is known or has yet to be identified. An attempt to answer these questions will be outlined in the next Chapter.

CHAPTER 5 : cDNA SEQUENCE ANALYSIS AND FERMENTATION PARAMETER EXPERIMENTS

5.1 INTRODUCTION

The previous Chapter described the isolation of sequences non-constitutively expressed during an anaerobic wine fermentation and linked their expression to phases of yeast growth. As outlined in the discussion, differences of several transcript types have been observed during the yeast life span. The first half of this Chapter examines the possible types of sequences isolated at each phase of growth with reference to the literature and indicates their association with a wine fermentation.

Originally two cDNA libraries were constructed from mRNA extracted of wine yeast during early and late stages of growth during an anaerobic wine ferment. The majority of transcripts in each library are expressed throughout the fermentation and are presumed necessary for cell life. However, a small proportion (approximately 1 percent) have been isolated that are associated with either stage of growth. It is expected that transcripts expressed during the exponential phase of growth represent genes that are associated with the growth and division of cells, whereas sequences expressed at stationary phase are associated with the stress response initiated to the continually changing environment. In the literature, much importance has been placed on gene expression related to stress responses such as temperature shock, accumulation of toxic products and nutrient depletion ultimately leading the cell to enter into a stationary phase of growth. However, often the major limiting nutrient in batch culture growth of laboratory yeast strains in aerobic conditions is glucose; a state not directly pertinent to wine fermentations. Therefore, this Chapter also attempts to look at the major regulatory parameters of a wine fermentation to determine the effects in the induction of gene expression and on transcript levels by altering specific fermentation conditions.

5.2 SEQUENCE DATA

During the course of the cross-hybridisation analysis (Chapter 4) some clones showed slight cross reactivity. Thus, sequence analysis was carried out, firstly to gain information concerning the possible functions of these genes, and secondly to investigate whether the clones represent different genes or pseudogenes.

Complementary DNA clones were subcloned into pTZ19U and the nucleotide sequences determined. These were then compared with sequences kept in nucleic acid and protein databases, such as Genbank, EMBL, PIR and Swiss-Prot. Most significant matches occurred with protein databases. An example of matches with 5 different cDNA clones from each library are provided in Table 5.1.

The table indicates the cDNA clone sequenced, its frequency amongst the clones isolated from each library (clonal family size), the sequence homology shown with the protein databases and the percent homology matched per amino acid number. Similarities were observed to proteins in the databases and will be discussed in more detail below.

5.2.1 "Early" cDNA Clones

All 5 cDNA clones sequenced from the "early" stage-specific library underwent cross-hybridisation studies to identify any relationship between the clones. Only slight cross reactivity was observed between the families of clones E50 and E105. Subsequently, sequence analysis was undertaken to determine their relatedness. All 5 cDNA sequences showed homology to different ribosomal proteins (rp) (Table 5.1).

cDNA clones E13 and E105 showed homology to duplicated 40S ribosomal proteins L34 (Schaap *et al.*, 1984) and S18 (Folley and Fox, 1994) respectively. Clone E126 showed homology to the acidic ribosomal protein L45 (Remacha *et al.*, 1988) which has been mapped to chromosome IV (Remacha *et al.*, 1990), cDNA E50 showed homology to the 60S ribosomal subunit URP1 (Jank *et al.*, 1993) and clone E135 showed homology to yeast ribosomal protein S12 (Moore *et al.*, unpublished data).

5.2.2 "Late" cDNA Clones

The "late" cDNA clones showed homology to a variety of genes (Table 5.1). Complementary DNA clone L30 showed homology to a gene *YGP1* which encodes a highly glycosylated secretory protein (gp37) (Destruelle *et al.*, 1994). The functional role of *YGP1* is not known. However, Destruelle's sequence of *YGP1* displayed homology to the sporulation-specific wall maturation protein gene (*SPS100*). This sequence was also the second on the list of database homologies for the L30 cDNA clone. Clone L13 showed homology to a gene *PIR3* first isolated by Toh-e *et al.* (1993). Toh-e *et al.* (1993) isolated three highly homologous genes *PIR1*, *PIR2* and *PIR3*, collectively called the *PIR* genes. The *PIR* genes have been shown to be required for tolerance to heat shock and also showed significant homology, along with the L13 cDNA, to a heat shock gene (*HSP150*) isolated by Russo *et al.* (1991a).

Table 5.1

Clone Number	Clone Family Size (% / clones isolated)	Sequence Homology	Percent Homology per Amino Acid Number	Reference
E13	4.9%	yeast ribosomal protein L34	100% in 44 aa of 113 aa	Schapp *et al.* (1984)
E50	3.5%	yeast ribosomal protein URP1	98% in 155 aa of 160 aa	Jank *et al.* (1993)
E105	6.9%	yeast ribosomal protein S18	100% in 86 aa of 155 aa	Folley and Fox (1994)
E126	5.6%	yeast ribosomal protein L45	100% in 85 aa of 110 aa	Remacha *et al.* (1988)
E135	6.9%	yeast ribosomal protein S12	94% in 97 aa of 99 aa	Moore *et al.* (unpublished data)
L13	5.1%	yeast *PIR3* gene	100% in 45 aa of 325 aa	Toh-e *et al.* (1993)
L20	2.2%	yeast hexose transporter *HXT7*	100% in 171 aa of 570 aa	Reifenberger *et al.* (1995)
L30	28.9%	yeast *YGP1* gene	100% 74 aa of 354 aa	Destruelle *et al.* (1994)
L49	19.6%	yeast *SED1* gene	85% in 48 aa of 338 aa	Hardwick *et al.* (1992)
L81	18.1%	yeast hypothetical protein	100% in 137 aa of 148 aa	Dietrich *et al.* (unpublished data)

Table 5.1 : Sequence homology of cDNA clones to sequences found within international data bases.

The "late" cDNA clone L20 showed homology to a hexose transporter gene (*HXT7*), located on chromosome IV, associated with the transport of glucose and fructose into yeast cells (Reifenberger *et al.*, 1995). Complementary DNA clone L49 showed homology to *SED1* isolated by Hardwick *et al.* (1992) who suggests this gene probably encodes a cell surface glycoprotein. Another match of a late cDNA clone (L81) included homology to an uncharacterised gene of the databases (sequence submitted by Dietrich *et al.*, unpublished data).

5.3 FERMENTATION PARAMETERS

In order to define the profiles of induction or repression of transcription of these sequences it is important to understand the regulating parameters governing the growth and metabolism of yeast under fermentation conditions. Possible major influences of yeast growth and metabolism in a wine fermentation are the high concentration of sugar, the increasing concentration of ethanol and the lack of oxygen. Other factors affecting growth and fermentation rates may include pH, temperature, original inoculum density and the limiting availability of nutrients.

In the high concentration of sugar and acid pH of the must, yeasts follow a specific cycle of growth and metabolism and fermentation activity is progressively inhibited by the products of sugar metabolism. That is, sugar and ethanol are involved in the inhibition of yeast growth: high concentrations of sugar in the must restrict the amount of growth and the accumulation of ethanol in the cell initiates the arrest of growth. Pure culture studies of *S. cerevisiae* show that the rate and completeness of fermentation decreases as the initial concentration of sugar in the juice increases above 200g/l. Such high concentrations affect yeast growth by increasing the lag phase, decreasing growth rate and decreasing the maximum population of cells produced (Lafon-Lafourcade, 1983; Monk and Cowley, 1984). The inhibitory action of ethanol produced in the course of fermentation is complex. Lafon-Lafourcade and Ribereau-Gayon (1984) have shown that inhibition of metabolism is linked to the production of toxic substances by yeast acting in synergy with ethanol. One of the major target sites of ethanol in yeast is the plasma membrane, as well as the membrane of the various cellular organelles (Thomas and Rose, 1979). The damage caused by ethanol to the cell membrane results in altered membrane organisation and permeability. The synthesis of cell wall membrane components requires molecular oxygen. Consequently, ethanol tolerance, ethanol yield and cell viability are positively influenced by the presence of a small amount of oxygen in the medium (Ryu *et al.*, 1984; Ingledew and Kunkee, 1985; Oliver, 1987).

The typical grape juice fermentation is anaerobic; the process of fermentation itself requires no oxygen. The yeast starter culture is grown aerobically, however, after inoculation, during the early stage of fermentation any available oxygen in the juice is consumed. Even in the presence of ample oxygen *S. cerevisiae* at high sugar concentrations preferentially ferment sugars. Nevertheless, as described above, trace amounts of oxygen favour fermentation, consequently, controlled aeration of the grape juice prior to fermentation or during the early stages of fermentation can stimulate yeast growth and fermentation performance (Ingledew and Kunkee, 1985).

Sugar, ethanol and oxygen represent three major parameters that are changing rapidly with the fermentation cycle. Other parameters, as mentioned below, can also effect yeast growth rate and fermentation performance, but within the limits of these microvinification trials are not changing dramatically and, as a consequence, should have a minor effect on the induction or repression of transcription.

The pH of grape juice varies between 3.0 and 4.0 depending on the concentrations of the two principal organic acids, tartaric and malic acids (Amerine and Ough, 1991). The pH has little effect on the rate of fermentation (Jackson, 1994), although a series of publications by Ough (1966a/b) has shown that low juice pH can prolong the initial lag period. Temperature has also been shown to be an important factor in wine fermentations and yeast kinetics. At warm temperature (>20^{0}C) yeast cells undergo a rapid decline in viability at the end of fermentation. At cooler temperatures, cell growth is retarded, but viability is enhanced. Cool temperatures also extend the lag phase of yeast growth (Jackson, 1994). Also Lafon-Lafourcade and Riberau-Gayon (1984) have shown that in inoculations varying from 10^4 to 10^6 cells per ml, the concentration of inoculum affects the speed of fermentation but not its completion.

5.3.1 Alteration of Fermentation Parameters

In order to define more precisely the profiles of induction or repression of transcription an experiment to determine the effect of changing the fermentation environment was carried out. Oxygen, ethanol and sugar concentrations were altered at specific times during wine fermentations and RNA was extracted for RNA hybridisation analysis.

Laboratory scale fermentations were prepared as described in Chapter 3, Section 3.4 with slight amendments as detailed below. An overnight culture of wine yeast strain AWRI 796, grown aerobically in YPD media, was inoculated in quadruplicate, into flasks of supplemented Sultana grape juice at a final concentration of approximately 5 x 10^6 cells/ml.

The must contained 190g/l fermentable sugars. One flask was allowed to undergo fermentation under anaerobic conditions at 18^0C with gentle agitation without alteration. The remaining three flasks were altered at specific stages of the ferment for oxygen, ethanol or sugar concentrations.

As was observed in the initial microvinification trials (Chapter 4), the shift from exponential to stationary phase and alterations in gene transcript expression occurred after approximately 40 hours of growth under fermentation conditions (Figure 4.2). "Early" stage-specific clones are strongly expressed until 40 hours with expression levels dropping after that time, whereas "late" stage-specific clones only begin expression after cells have entered stationary phase (T4-42hrs). At this stage ethanol concentration has risen to about 2 percent and sugar levels decreased to approximately 140g/l.

Alterations of fermentation flasks were subsequently undertaken to mimic the changing conditions of the ferment. That is, in one flask, ethanol was added to a final concentration of 2 percent within the first 12 hours of fermentation to mimic the levels observed at 40 hours. This may indicate, by altered transcription patterns, if any "early" stage-specific clones are repressed or "late" stage-specific clones induced by ethanol. In another flask after approximately 40 hours of growth equal amounts of glucose and fructose were added to lift the fermentable sugar levels back to the original concentration at the start of fermentation. Again this aims to show if any "early" stage-specific clones are induced or "late" stage-specific clones repressed due to high sugar concentrations. Ethanol and sugar fermentations were carried out under anaerobic conditions at 18^0C with gentle agitation.

An aerobic fermentation was also conducted. Prior to inoculation the supplemented grape juice for the aerobic ferment was not sparged with nitrogen. Instead, the flask was constantly sparged with air, using a fish tank aerator, prior to and during fermentation and agitated at 18^0C. This may indicate if any samples are expressed due to the dramatic change form aerobic starter culture conditions to the anaerobic fermentation environment.

Samples were taken from each flask at regular intervals and assayed for yeast growth (cell count; Figure 5.1a) and the progress of fermentation was monitored by sugar concentration, as change in specific gravity, (refractive index; Figure 5.1b), and ethanol concentration (% v/v; Figure 5.1c). The readings for each assay were plotted over time. RNA was also extracted at 12 hourly intervals for RNA hybridisation analysis.

Figure 5.1 : Comparison of fermentations altered for oxygen, ethanol and sugar levels, as described within the text, with a control fermentation, as monitored by

a) *Saccharomyces* Cell Growth

b) Sugar Utilisation (Refractive Index, ^{0}Brix)

c) Ethanol Concentration (% v/v)

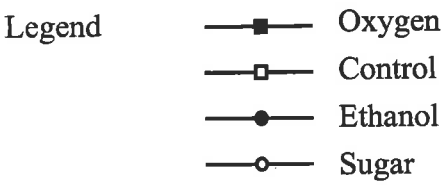
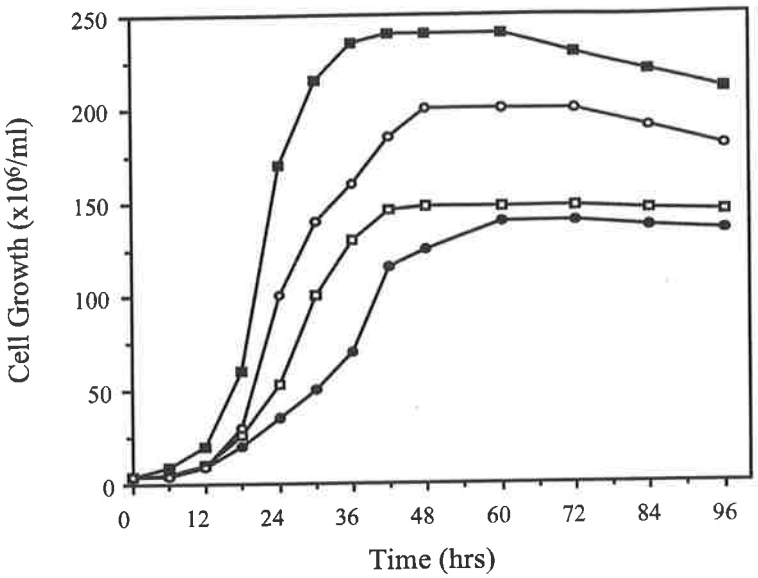
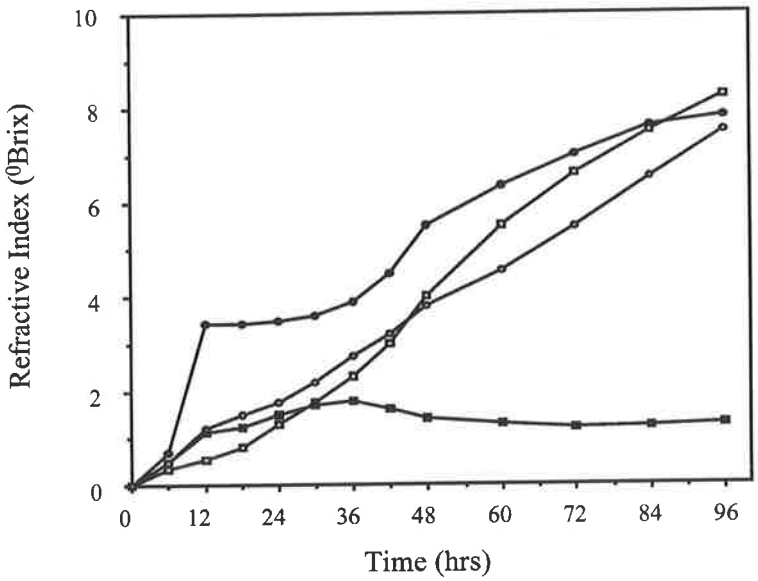


Figure 5.1

a)



b)



c)

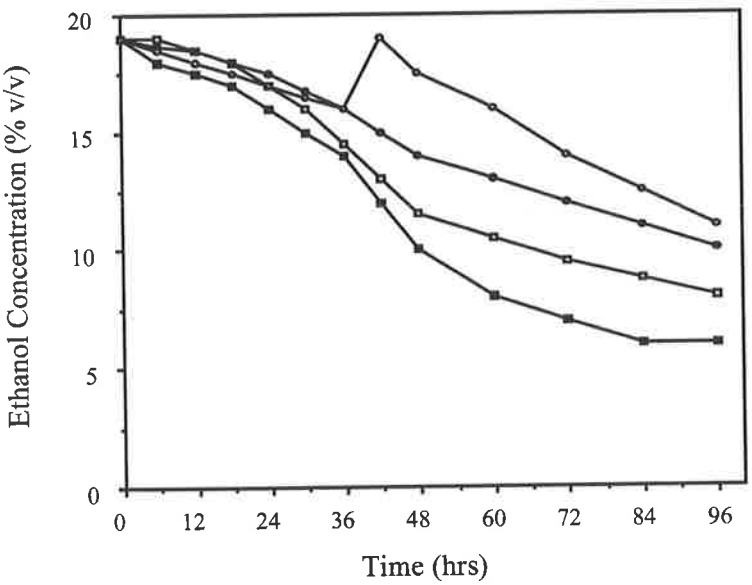


Figure 5.1a indicates the alterations in yeast growth between the different fermentation flasks. The control fermentation represents the standard pattern of yeast growth, entering stationary phase between 40 and 46 hours and persisting till the end of the fermentation with a total cell count of 1.4 x 10^8 cells/ml. Comparatively, yeast growth under aerobic conditions is rapid, reaching stationary phase at 36 hours at a total cell concentration of 2.4 x 10^8 cells/ml. Stationary phase is short and cell decline is dramatic. Both ethanol and sugar fermentations display a longer exponential period, reaching stationary phase at approximately 48 hours. Growth in the sugar fermentation flask appears to be bolstered leading to a final concentration of 2.0 x 10^8 cells/ml, where as, growth of cells within the ethanol fermentation flask exhibit slight growth inhibition, reaching a final concentration of 1.3 x 10^8 cells/ml.

5.3.2 RNA Hybridisation Analysis

RNA hybridisation analysis was carried out to determine changes in timing and level of expression of cDNAs due to changes in the fermentation parameters (Figures 5.2 and 5.3).

RNA hybridisation filters of each of the four fermentation flasks were hybridised with the same probe preparations (in excess) and all are related to the same preparation of an internal control (actin mRNA). Consequently, it is possible not only to detect changes in the induction or repression of transcripts between filters but also to compare quantitative differences in the abundance of the transcripts examined. In most cases, similar patterns of activation were observed. These are shown to be in the most part directly related to cell growth. The actin mRNA level, used as a reference, also appears to be growth dependent.

The variations observed, in general, were slight in terms of induction and repression with the timing of expression only shifting slightly with the yeast growth cycle. This is especially obvious with the two "early" cDNAs clones (Figure 5.2). For both clones E126 and E135 strong expression is observed in the first 36 hours (T1-T3) of the control ferment. Within the aerobic ferment this expression is reduced to 24 hours (T1-T2). The actin control also shows a dramatic reduction in expression, possibly due to the entrance of the cells into decline phase at an early stage (T5-60hrs). The transcript levels during the ethanol fermentation are similar to the control. Although, the exponential phase of growth is extended in yeast cells of this flask (Figure 5.1a), growth and consequently, transcription appears to be slightly restricted by the ethanol levels. Within the sugar fermentation flask transcription of the clones is observed up to T4 (48hrs) and matches perfectly with the growth curve of the cells (Figure 5.1a).

Figure 5.2 : RNA hybridisation analysis of "early" stage-specific clones under varying fermentation conditions.

Filters contain RNA extracted, at 12 hourly intervals, from yeasts undergoing fermentation: T1 -12hrs, T2 - 24hrs, T3 - 36hrs, T4 - 48hrs, T5 - 60hrs, T6 - 72hrs.

Complementary cDNA clones used as probes are a) E126 and b) E135.

Fermentation conditions as outlined in the text are:

i) control - no alteration

ii) aerobic - sparged with air prior to and during fermentation

iii) ethanol - addition of ethanol to a final concentration of 2% within the first 12hrs.

iv) sugar - addition of sugar to a final concentration of 190g/l at 40hrs.

Actin control filters for each hybridisation pattern are also displayed.

Figure 5.2

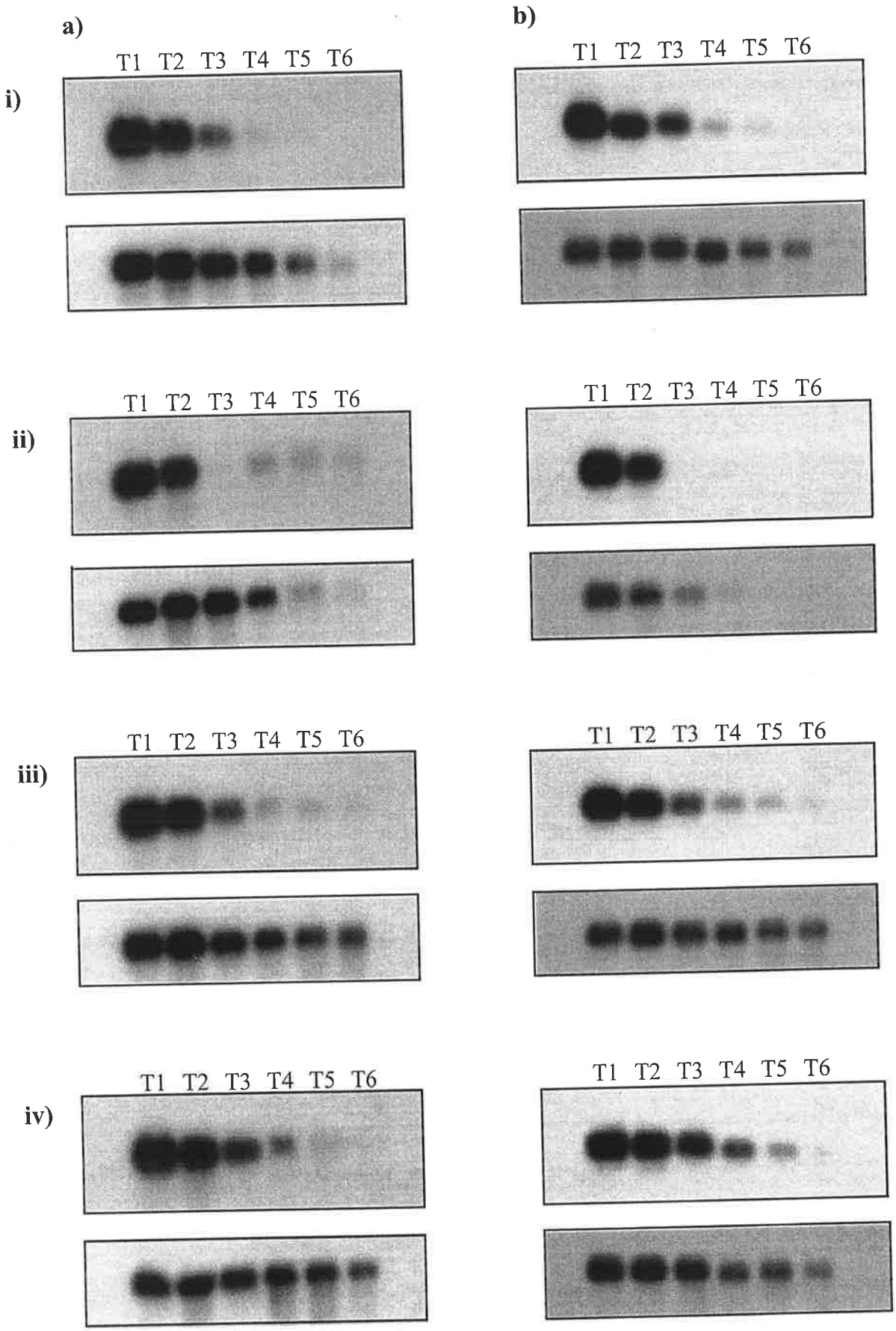


Figure 5.3 : RNA hybridisation analysis of "late" stage-specific clones under varying fermentation conditions.

Filters contain RNA extracted, at 12 hourly intervals, from yeasts undergoing fermentation: T1 - 12hrs, T2 - 24hrs, T3 - 36hrs, T4 - 48hrs, T5 - 60hrs, T6 - 72hrs.

Complementary cDNA clones used as probes are a) L20 and b) L13

Fermentation conditions as outlined in the text are:

i) control - no alteration

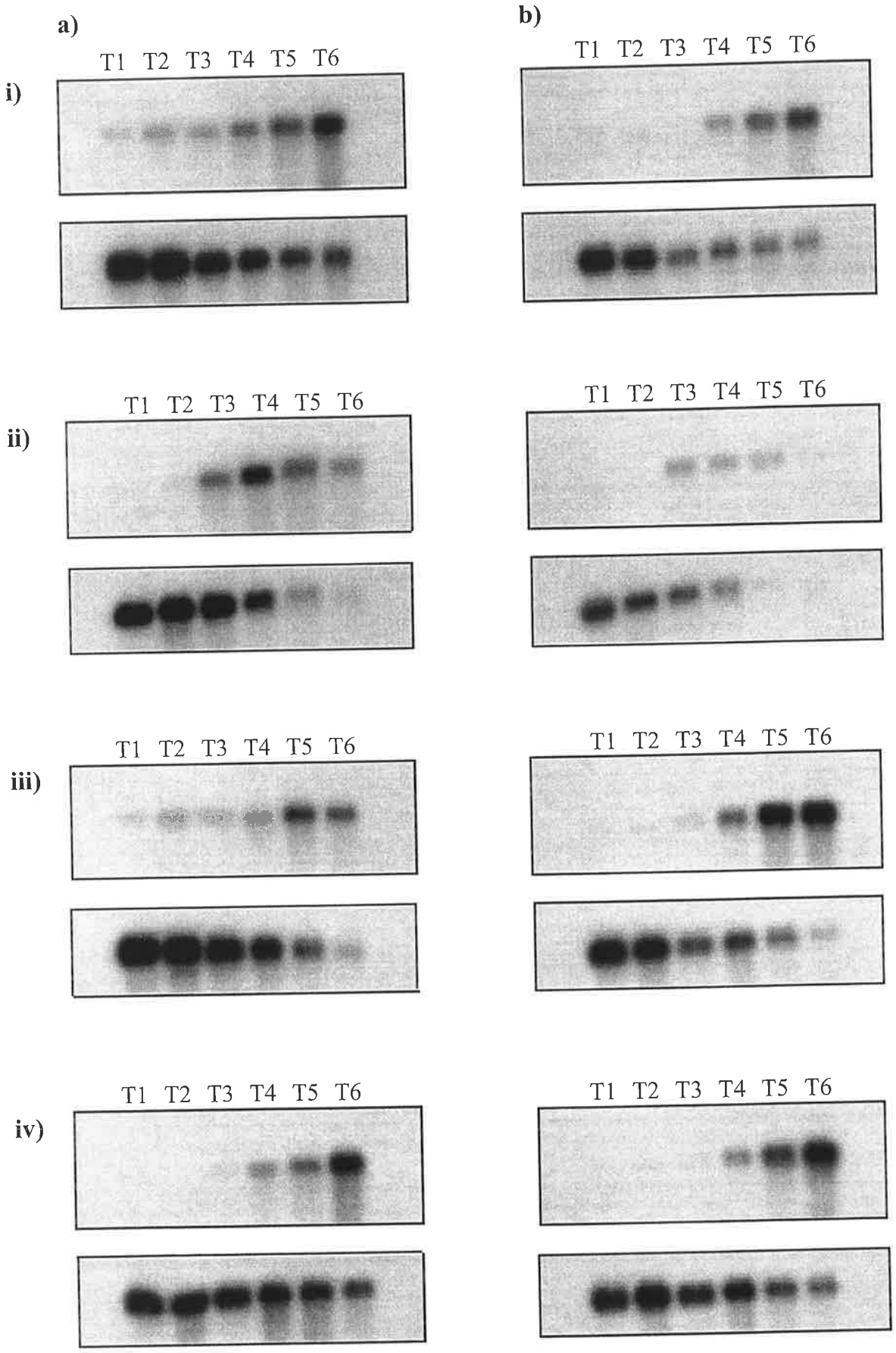
ii) aerobic - sparged with air prior to and during fermentation

iii) ethanol - addition of ethanol to a final concentration of 2% within the first 12hrs.

iv) sugar - addition of sugar to a final concentration of 190g/l at 40hrs.

Actin control filters for each hybridisation pattern are also displayed.

Figure 5.3



A similar change in expression timing is observed with both "late" stage-specific clones examined. However, the most interesting result concerns the relative abundance of the transcripts and their variation among the different fermentation conditions: occasionally dramatic quantitative differences in the abundance of transcripts was observed (Figure 5.3).

Analysis of the L20 clone in the control ferment shows low level expression until T4 (42hrs) with transcripts levels increasing until the last extraction at 72 hours (T6) (Figure 5.3a). Expression within the aerobic ferment shows no basal expression and transcription is observed to correlate with cell growth. That is, expression begins at 36 hours (T3) as cells enter stationary phase (Figure 5.1a). Expression within the ethanol fermentation flask is similar to the control, although levels do appear to be decreased. The sugar fermentation shows no basal expression and strong transcription only begins as cells enter stationary phase (T5-54hrs).

The L13 control ferment displays a model expression pattern characteristic of stationary phase expression (Figure 5.3b). A shift in expression in the aerobic ferment with the growth cycle is again observed but also a severe decrease in the amount of transcript is detected. A similar expression pattern as the control is seen within both the ethanol and sugar ferments, although transcript levels are increased.

5.4 DISCUSSION

In this Chapter several cDNA clones were sequenced and the putative amino acid sequence for the protein was determined. These sequences were compared via international databases with others from both eukaryotic and prokaryotic sources. All "early" stage-specific clones show strong homology with ribosomal proteins. This is not an unusual result as there is evidence that ribosomal protein expression is tightly linked to the growth cycle of yeast cells (Kief and Warner, 1981; Planta *et al.*, 1986; Donovan and Pearson, 1986; Kraakman *et al.*, 1993; Ju and Warner, 1994).

Ribosomes are ubiquitous in living cells and play a central role in cell growth and maintenance. Ribosomes are composed of a complex mixture of RNA and protein molecules which are arranged in an orderly array in order to produce a biologically active particle. In the yeast ribosome there are four species of rRNA (25S, 18S, 5.8S and 5S) and approximately 75 ribosomal proteins. The number of ribosomal proteins has not been unequivocally assigned, but standard nomenclatures have been proposed by Bollen *et al.* (1981) based on 32 proteins in the 40S subunit and 45 proteins in the 60S subunit.

Originally it was shown, in bacteria by Maaloe and Kjeldgaard (1966) that during exponential phase, the cells content of ribosomes is proportional to its growth rate when growth is limited by carbon or nitrogen sources. Subsequently, a number of studies have shown that in yeast *S. cerevisiae* a similar situation occurs (Waldron and Lacroute, 1975; Warner, 1982). Furthermore, in a nutritional shift-up experiment, when a grown culture was enriched with glucose, cells responded with a preferential stimulation of the synthesis of both ribosomal RNA and proteins (Kief and Warner, 1981). Indeed many researchers have shown coordinate expression of ribosomal protein genes in yeasts. This occurs primarily at the transcriptional level, as a function of cellular growth rate (Kief and Warner, 1981; Planta *et al.*, 1986; Donovan and Pearson, 1986; Kraakman *et al.*, 1993; Ju and Warner, 1994). Also, the relative cellular levels of various individual rp-mRNAs have been shown to be approximately equal under a variety of conditions (Donovan and Pearson, 1986; Mager, 1988; Mager and Planta, 1991).

Comparison of cDNA clone family sizes as indicated in Table 5.1 show that similar levels of ribosomal protein transcripts were isolated during the differential screening process. Analysis of RNA hybridisation filters indeed shows expression to be coordinated and linked to cell growth; expression begins with the lag phase, persists through the exponential phase and disappears upon entry into stationary phase.

As cells enter stationary phase the transcription of most genes and the level of mRNA decreases (Destruelle *et al.*, 1994). Similarly there is a substantial drop in overall protein synthesis (Boucherie, 1985). In contrast, some genes are expressed at higher levels (Werner-Washburne *et al.*, 1993). Most of these gene products appear to be required for survival under stress conditions. Two well documented changes are resistance to killing by heat, with the expression of heat shock response genes (Schenberg-Frascino and Moustacchi, 1972; Walton *et al.*, 1979; Plesset *et al.*, 1987), and the strengthening of the cell wall to prevent degradation by enzymes and toxic products (Deutch and Parry, 1974; Zlotnik *et al.*, 1984; DeNobel *et al.*, 1990a/b).

In *S. cerevisiae* several researchers suggest a relationship between heat shock proteins and nutritional limitation (Boorstein and Craig, 1990; Susek and Lindquist, 1990; Pillar and Bradshaw, 1991; Praekelt and Meacock 1990; Russo *et al.* 1991a). For example, the small heat shock protein gene (*HSP26*) of *Saccharomyces cerevisiae* is not expressed in unstressed growing cells but is strongly induced during heat shock, stationary phase growth and early after transfer to sporulation medium (Kurtz and Lindquist, 1984; Kurtz *et al.*, 1986; Susek and Lindquist, 1990).

A common feature of the sequenced "late" cDNA clones is their homology to either cell surface or heat shock proteins. Characterisation of these genes by researchers has shown similar expression patterns and suggest expression at the stationary phase of growth. For example, the *YPG1* gene, encoding the highly glycosylated secretory protein (gp37) matched to clone L30, has been sequenced and the regulation of its expression examined (Destruelle *et al.*, 1994). They found that *YGP1*-specific RNA is not expressed at high levels in cells during exponential growth but expression is increased in response to the limitation of various nutrients. Destruelle *et al.* (1994) suggests that the *YGP1* gene is subject to glucose repression because their experiments indicate a 50 fold increase in expression below 1 percent glucose. A nutrient depletion not observed during wine fermentations. However, derepression of *YGP1* was also seen when cells were grown under conditions in which phosphate or nitrogen became limiting. Destruelle *et al.* (1994) also observed that different types of nutrient limitation led to various levels of protein synthesis suggesting the involvement of multiple regulatory elements.

Clone L13 showed homology to the gene *PIR3*. Toh-e *et al.* (1993) showed that the *PIR* genes are necessary for tolerance to heat shock. Disruption of individual genes did not confer sensitivity to heat shock on the cell due to the redundancy of the *PIR* genes. However, double disruptants showed a clear heat shock sensitive phenotype. The *PIR* genes produce extracellular proteins secreted into the medium which Toh-e *et al.* (1993) proposed to function by protecting heat shocked cells from without.

Clone L20 showed homology to a yeast hexose transporter *HXT7*. In *S. cerevisiae* there are at least five genes which encode putative glucose transporters. For reviews refer to Bisson *et al.* (1993) and Lagunas (1993). The transport of glucose and fructose into the cells of the yeast *S. cerevisiae* has been well characterised kinetically (Bisson and Fraenkel, 1983a/b, 1984; van Urk *et al.*, 1989; Ko *et al.*, 1993). Two distinct transport systems have been described, one with low affinity and the other with high affinity for glucose. The low affinity system is constitutive, whereas the high affinity system is repressed at high extracellular glucose concentrations (Bisson and Fraenkel, 1984; Bisson, 1988). Reifenberger *et al.* (1995) showed that *HXT7* expression was inhibited by glucose levels above 1 percent indicating a high affinity system. Consequently, it is anticipated that its expression pattern within a wine fermentation would match that seen by clone L20.

In an attempt to elucidate the mechanisms of regulation of clones isolated, experiments were carried out to investigate gene expression under different conditions. RNA hybridisation analysis of "late" clones was conducted to examine the levels of induction and repression of cDNA expression by alteration of the major fermentation parameters.

In general, expression was coordinated with yeast growth; strong expression beginning as cells enter stationary phase. Differences were also observed in the level of transcripts produced (especially for L13). Given that the clone L13 can not be conclusively designated a *PIR* gene and the function of PIR proteins is still unknown, it is difficult to speculate about the effect on regulation by these parameter changes. However, it is interesting, to note that the differences observed, due to changes in the ethanol and sugar levels, are likely to only indicate a small proportion of possible potential regulatory events.

Addition of exogenous substances does not truly mimic the extensive changes occurring within the fermentation cycle. For example, it has been shown that exogenous ethanol added to *S. cerevisiae* is less toxic than endogenous ethanol produced by the yeast (Thomas and Rose, 1979; Loureiro and Ferreira, 1979). Thus the effect on gene transcription by exogenous ethanol addition does not truly reflect expression of the yeast under fermentative conditions. Also, it has been shown that the expression of genes whose products play a role in stress adaptation are often regulated in a manner that allows the cell to sense and respond to changing environmental conditions. For example, Destruelle *et al.* (1994) has shown that the *YPG1* gene is derepressed 50 fold as medium glucose falls below 1 percent glucose. However, the same expression pattern is not observed when cells are transferred to 1 percent. That is, the glucose-sensing mechanism does not operate when cells are directly shifted to low glucose concentration. These results suggest that *YGP1* may be regulated through a complex mechanism that senses and responds to multiple environmental signals and that the gene product may play a role in cellular adaptations prior to entry into stationary phase.

Although only a crude experiment was conducted into the regulatory parameters of sequences it is important to note that for many sequences their expression is coordinated with the yeast cell growth cycle. As a result, the promoter sequences of these genes have the potential to carry out stage-specific expression in a variety of fermentation conditions: expression of genes controlled by these promoters will be coordinated to the phases of yeast growth. Variation of fermentation conditions from wine to wine and winery to winery should not effect this regulated expression.

CHAPTER 6 : SELECTION OF CLONES FOR PROMOTER ISOLATION

6.1 INTRODUCTION

This Chapter describes experiments to investigate the expression pattern of commonly used yeast promoters under the conditions of an anaerobic wine fermentation. The promoters which were tested for constitutive expression include alcohol dehydrogenase (*ADH1*) and phosphoglycerate kinase (*PGK1*), and for regulated expression, acid phosphatase (*PHO5*). Comparison of these expression patterns to those of the stage-specific cDNA sequences isolated in Chapter 4 is also discussed. Finally, the selection of two cDNA sequences for detailed characterisation of the corresponding genes and isolation of their 5' non-coding sequences for trial as putative promoter elements is outlined.

6.2 COMMONLY USED YEAST PROMOTERS

Both constitutive and regulated promoters have been used to express recombinant proteins in laboratory and industrial yeast strains. The most frequently used promoters are those related to genes encoding abundantly expressed glycolytic enzymes, such as the genes for alcohol dehydrogenase (*ADH1*), phosphoglycerate kinase (*PGK1*) or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Ammerer, 1983; Tuite *et al.*, 1982; Bitter and Egan, 1984). Although expression from promoters of these genes is essentially constitutive it has been observed that there is some fluctuation in the level of expression depending on growth conditions (*ADH1*: Denis *et. al.*, 1983; *PGK1*: Chambers *et al.*, 1989; Moore *et al.*, 1991). Denis *et al.* (1983) showed that ADH1 mRNA and the rate of ADH1 protein synthesis decreases upon growth in ethanol containing medium and during growth into stationary phase. Hence, as the *ADH1* promoter is often used in the synthesis of foreign proteins in industrial yeast strains it seemed important to establish an expression pattern for *ADH1* during wine fermentations. Analysis was also carried out on the constitutive *PGK1* gene.

Many regulatable promoters rely on the addition or removal of compounds to induce or repress expression, but this is often not compatible with the oenological process. Commonly used regulatable promoters in the laboratory are often glucose repressible and require the depletion of glucose and the addition of a replacement carbon source to induce expression. For example, the *GAL1*, *GAL7* and *GAL10* promoters are repressed in cells grown in the absence of galactose or in the presence of glucose. They are induced 1000 fold when galactose is present and glucose absent (St John and Davis, 1981b). The *ADH2* regulatable promoter also relies on the depletion of glucose for derepression and

expression (Price *et al.*, 1990). Alternatively, the promoter of the *CUP1* gene encoding copper metalliothonien is tightly regulated independently of culture parameters. However, induction depends on the addition of copper ions to the culture (Etcheverry, 1990). Consequently, these systems are inappropriate for use in commercial wine making because of the high sugar concentration present in must and constraints concerning alteration of product composition.

One regulatable promoter that may be potentially applicable to fermentations, as it shows tight on/off control in response to changing inorganic phosphate concentration, is the *PHO5* promoter. The *PHO5* gene encodes repressible acid phosphatase (Bostian *et al.*, 1980b; Meyhack *et al.*, 1982) and is controlled by a complex but genetically well defined system (reviewed by Toh-e, 1989; Vogel and Hinnen, 1990). A number of heterologous proteins have been successfully expressed in *S. cerevisiae* by using the *PHO5* promoter, including hepatitis B surface antigen (Miyanohara, *et al.*, 1983) and α-antitrypsin (Rosenberg, *et al.*, 1984). More recently, the use of *PHO5* and *PHO5* derived promoters for regulated heterologous expression has been developed by Hinnen *et al.* (1989). The basis for this work was founded on a detailed analysis of the *PHO5* promoter elements by deletion mapping and promoter fusions (Bajwa *et al.*, 1987; Rudolf and Hinnen, 1987).

PHO5 is not transcribed at a detectable level in yeast growing in media containing inorganic phosphate. However, when inorganic phosphate is depleted from the medium, transcription of *PHO5* is induced to yield a level of mRNA which may account for up to 5 percent of the total cellular polyadenylated RNA (Oshima, 1982). Consequently, when using the *PHO5* promoter in a laboratory expression system the medium can be designed such that inorganic phosphates are depleted when the yeast strain reaches a high density. Although manipulation of grape must is generally undesirable it may be possible, given the deficiency of nutrients in must as compared to laboratory media, that inorganic phosphates would decrease to derepression levels as a natural course of the fermentation. The phosphate content of must, present in both inorganic and organic forms varies from about 50 to 900mg/l depending on the grape source (Amerine and Ough, 1991). Hence, *PHO5* may be a suitable late inducible promoter for use in wine fermentations.

6.2.1 RNA Hybridisation Analysis

RNA filters used in the expression studies of isolated cDNA clones (Chapter 4) were stripped of any annealing probe and rehybridised with radio-labelled sequences of common yeast promoter genes *ADH1*, *PGK1* and *PHO5*. Filters contained RNA extracted at regular intervals (every 12hrs for the first 72hrs) from yeast during an anaerobic

fermentation. DNA probe sequences for the identification of expression of each gene were obtained by amplification from genomic DNA by PCR. Sequence information for the genes *ADH1*, *PGK1* and *PHO5* was obtained from the international databases and a computer based oligo program used to develop primers for their amplification. The experimental method, primer sequences and PCR programs are outlined in Chapter 3.

Figure 6.1 shows the expression pattern for each gene during fermentation. Both *ADH1* and *PGK1* probes detect transcripts at each extraction time of the fermentation indicating constitutive expression. Contrary to expectations, however, *PHO5* displayed an expression pattern opposite to that observed in batch cultures. The expression of *PHO5* is induced by the depletion of inorganic phosphates within the culture medium. Hence, in laboratory batch cultures expression is not normally observed until late in the growth cycle when all phosphates are removed. The hybridisation of the *PHO5* probe to RNA from an anaerobic wine ferment shows strong expression of *PHO5* during the first 12 hours with basal expression persisting till the end of the ferment.

It is not always possible to take a promoter regularly used in laboratory conditions and expect the same performance under fermentation conditions. Consequently, it is desirable to find promoters regulated by fermentation parameters such that an expected expression pattern may be obtained. The following section describes the selection of two differentially expressed sequences of a wine fermentation for isolation of their promoters.

6.3 SELECTION OF cDNA CLONES FOR PROMOTER ISOLATION

For each cDNA library constructed, as described in Chapter 4, a large number of sequences have been isolated that are shown to be differentially expressed during an anaerobic wine fermentation. A description of the range of expression patterns observed is outlined in Section 4.4.4.

In most situations it is desirable to use a strong promoter for high expression of a heterologous gene. However, often the gene products are only required for a limited time and basal or overexpression may result in difficulties. Therefore, it is essential to have a regulated promoter with the highest possible difference between induced and non-induced states. That is, the system should have no or very low basal transcription levels under repressing conditions and very high transcriptional rates under derepressing or inducing conditions. Given these criteria, a single sequence from each expression library was then selected for isolation of its 5' non-coding region as use for possible putative promoters for stage-specific expression during wine fermentations.

Figure 6.1

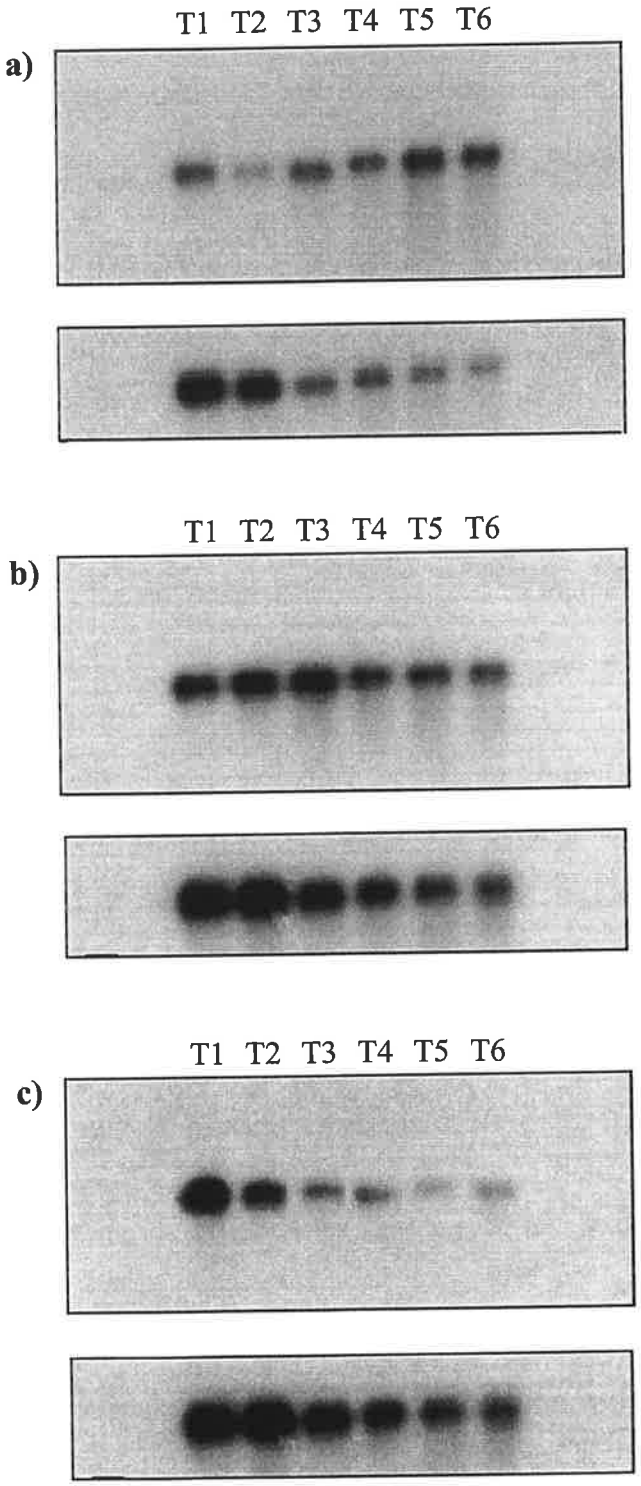


Figure 6.1 : RNA hybridisation analysis of commonly used yeast promoter genes.

Filters contain RNA extracted from anaerobic wine fermentations at 12 hourly intervals (T1 - 12hrs, T2 - 24hrs, T3 - 36hrs, T4 - 48hrs, T5 - 60hrs, T6 - 72hrs) and probed with radio-labelled a) *ADH1*, b) *PGK1* and c) *PHO5* sequences.

Actin control filters for each hybridisation pattern are also displayed.

Figure 6.2 demonstrates the expression patterns of the "early" and "late" cDNA clones that were chosen for further study. Many "early" cDNA clones, showing the same expression pattern, were identified by sequence analysis to be ribosomal proteins (Table 5.1). Further information garnered from the literature and outlined in the discussion below was used to identify the E126 "early" cDNA as the most appropriate clone for further work. The "late" stage-specific cDNA clone (L49) showed some sequence homology to the *SED1* gene (Table 5.1). However, sequence analysis of the genomic clone (described in Chapter 7) indicated that the clone represents an uncharacterised gene.

Clones were also picked based on their estimated level of expression. It is predicted that each mRNA sequence is represented in the cDNA expression library in proportion to their abundances in the original preparation of mRNA. Consequently, the size of the clone family within each expression library is suggested to be representative of the level of expression for that sequence. The size of each clone family within each library is indicated in Table 5.1. cDNA clone E126 shows homology to 6 percent of the "early" sequences isolated and the sequence of cDNA L49 is represented by 12 percent of the "late" clones isolated. As each clone is characterised by a large clonal family it suggests that each is expressed at abundant levels.

6.4 ISOLATION OF GENOMIC SEQUENCES

Recovery of a functional promoter sequence from the genome relies upon its tight linkage 5' to the transcription start site of the gene with the desired expression pattern. Consequently, a genomic clone for each cDNA was required to isolate the promoter sequences.

Yeast DNA was partially digested with Sau3A and size fractionated by sucrose gradient centrifugation. Fractions containing DNA fragments in the approximate size range of 15kb to 20kb were pooled and the DNA was ligated to λgem 11 arms. The recombinant phage were packaged and plated as outlined in Chapter 3. Duplicate plaque lift filters of the genomic library were hybridised with the "early" and "late" cDNA clones, respectively. The "early" cDNA clone hybridised to 11 genomic clones while the "late" cDNA clone identified 5 genomic clones. A genomic clone for each probe was isolated as a pure phage lysate and used in further analysis.

Figure 6.2

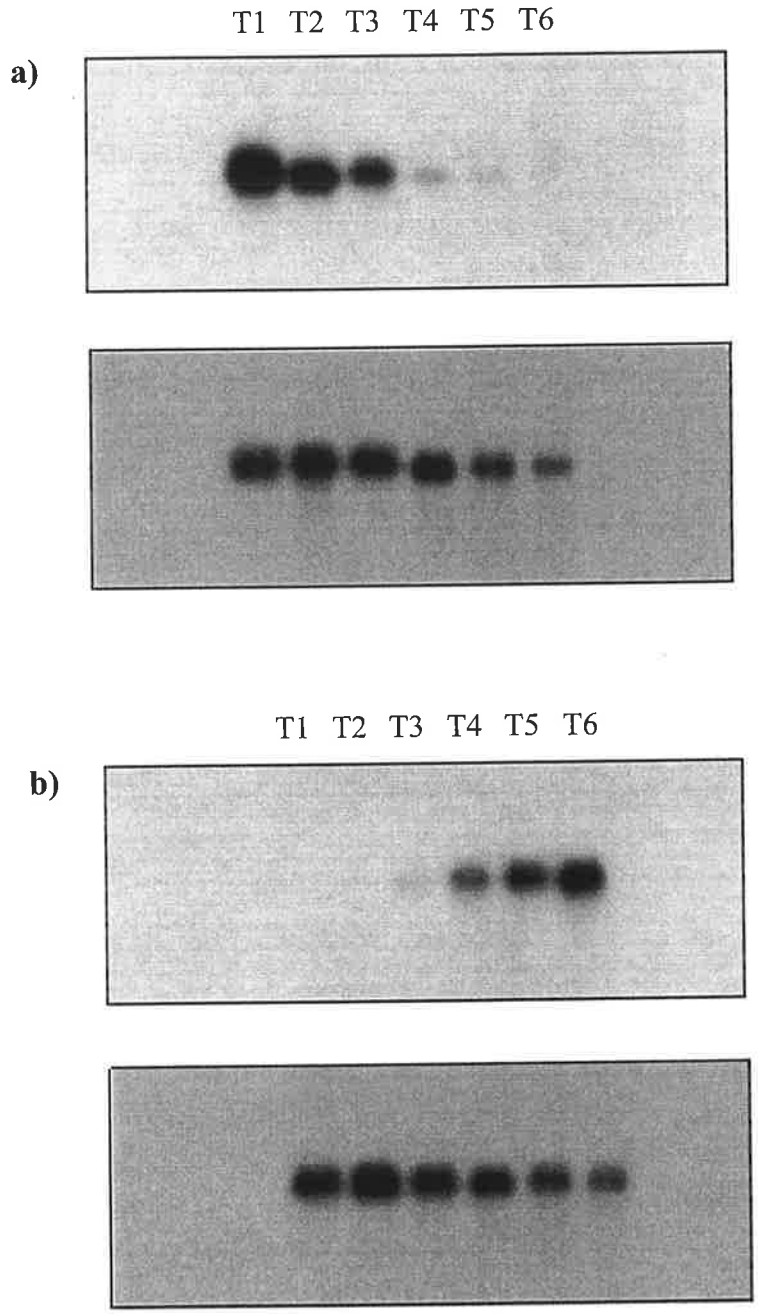


Figure 6.2 : RNA hybridisation analysis of a) "early" stage-specific clone E126 and b) "late" stage-specific clone L49.

Filters contain RNA extracted at 12 hourly intervals from yeasts undergoing fermentation.

Extraction times; T1 - 12hrs, T2 - 24hrs, T3 - 36hrs, T4 - 48hrs, T5 - 60hrs, T6 - 72hrs.

Actin control filters for each hybridisation pattern are also displayed.

6.5 DISCUSSION

It has often been reported that commonly used promoters in laboratory yeast are not applicable for use in industrial situations (Gossens *et al.*, 1993; Barre *et al.*, 1993; Watari *et al.*, 1994b; Vakeria and Box, 1996). Frequently, promoters chosen originally for their strong constitutive expression have negative effects on the expression of the heterologous gene or the observed expression patterns are contradictory to those expected. Consequently, it is not always possible to take a promoter regularly used in batch culture and expect the same performance under fermentation conditions.

Analysis of the expression of some commonly used promoters of laboratory yeast were examined during wine fermentations. Firstly, it was shown that the strong glycolytic *ADH1* and *PGK1* promoters do indeed show high level constitutive expression during anaerobic wine fermentations (Figure 6.1). Consequently, the use of these promoters in the genetic manipulation of wine yeasts may be compatible with the oenological process if strong continual expression is desired. Although, in some circumstances the *PGK1* promoter has been shown to under perform when used to express heterologous proteins; the extent to which it under performs depends on the particular protein which is being produced (Mellor *et al.*, 1985; Vakeria and Box, 1996). Therefore, when using these promoters each new gene to be expressed will need to be evaluated independently and assessed individually for optimisation of the expression conditions.

As described previously, many regulatable promoters are glucose repressible or rely on the addition of exogenous substances for induction. These are inapplicable for use in wine fermentations because of the high glucose levels (never decreasing enough for derepression) and the constraints involving product alteration. One promoter not restricted by these constraints and consequently potentially applicable to fermentations is the *PHO5* promoter. However, analysis of *PHO5* expression during an anaerobic fermentation of grape must produced an expression pattern contrary to expectations. Instead of an absence of expression early in the cycle and induction with the depletion of phosphates, as observed in laboratory conditions, strong expression was observed at the beginning of the ferment with low level expression persisting until the end (Figure 6.1).

A possible explanation for this observed expression pattern may be that before inoculation, cells were grown in YPD to stationary phase. Therefore, cells came from a medium where inorganic phosphates were depleted and transcription of *PHO5* derepressed. Inoculation with cells already containing transcripts could account for the detection of *PHO5* mRNA

early in the ferment. Another anomaly arises from the low level of expression observed throughout the ferment. In laboratory conditions, *PHO5* has been shown to have tight on/off control without leakiness or basal level expression (Bostian *et al.*, 1980b; Meyhack *et al.*, 1982). A possible explanation involves the conditions of the fermentation. The regulatory mechanism for *PHO5* expression is found to be disturbed at acidic pH; cells grown at pH 3.0 in low phosphate medium lack acid phosphatase activity. However, acid phosphatase activity is increased immediately upon shifting the pH of the medium to 4.0 or above (Toh-e *et al.*, 1978; Oshima, 1982). Wine fermentations are acidic environments. The original pH of the ferment from which the hybridisation filters were produced was 3.5 and a decrease to around pH 3.2 was observed within the first 30 hours. It may be that appropriate levels of inorganic phosphate are present for *PHO5* expression, however fermentation pH parameters inhibit this.

As demonstrated above, enhancing expression levels of proteins within fermentation conditions is a complex task and numerous parameters must be considered. It is now recognised that the choice of promoter is extremely important. Promoters must be found that are compatible with the commercial production of one particular product, as well as satisfying the economic and logistic factors involved. In terms of the wine industry this concerns the development of promoters for gene regulation that depend on parameters consistent with wine fermentations. Consequently, two sequences were chosen, from fermentation expression libraries, based on their expression pattern during a wine fermentation for use as potential modules for gene regulation under oenological conditions.

As described in Chapter 5 all "early" stage-specific cDNA clones sequenced were shown to represent ribosomal protein genes (rp-genes). Ribosomal protein genes have been extensively studied in recent years with about 40 rp-genes cloned and analysed. Comprehensive reviews include Planta *et al.* (1986), Raue and Planta (1991), Lee (1991) and Mager and Planta (1991). The extensive information available aided in the selection of the most appropriate ribosomal protein clone for the isolation of its promoter. The rational used to select the ribosomal clone for further work is outlined below.

Firstly, an important factor to consider in the choice of the promoter sequences of ribosomal protein genes is that most mRNAs for these proteins are encoded by duplicate genes. In these cases, the contribution of the individual gene copies to the final amount of mRNA is not necessarily equal (Fried *et al.*, 1981). For example, two *RP51* gene copies produce 40 and 60 percent of the *RP51* mRNA (Abovich and Rosbash, 1984) while for *L16* (Rotenberg *et al.*, 1988) and *RP28* (Herruer *et al.*, 1987) genes, this ratio is about

30:70 and 15:85, respectively. This effect has been clearly shown, by the above researchers, to be due to a difference in transcriptional activity at the respective genes. Hence, selection of a rp-gene promoter that occurs in duplicate within the genome may not produce the expected expression strength.

Another feature to consider is the presence of introns in many ribosomal protein genes. The common occurrence of an intron in rp-genes has led to speculation that they may play a regulatory role in protein synthesis, although, only one instance of regulation at this level has been described (Dabeva, *et al.*, 1986). Nevertheless, choice of an intronless rp-gene will ensure that gene expression is regulated purely at the upstream promoter.

A set of very acidic proteins is a general feature of ribosomes from all organisms (Vidales *et al.*, 1981a/b; Bielka, 1982). The acidic ribosomal proteins of yeast are encoded by a single gene (Remacha *et al.*, 1988). The yeast ribosome contains three acidic proteins L44', L44 and L45 (Sanchez-Madrid *et al.*, 1979, Vidales *et al.*, 1984; Remacha *et al.*, 1988). The genes for acidic proteins L44' and L45 have been mapped to chromosomes IV and L44 to chromosome XV (Remacha, *et al.*, 1990). Also distinguishing L45 and L44 from L44' and other ribosomal proteins is their lack of an intron commonly observed at the 5' end of most rp-genes.

Within the "early" stage-specific clones described in Chapter 4, a family of cDNA clones were shown to have homology with the acidic ribosomal protein L45. Although the sequence for rp-gene *L45* has already been characterised by Remacha *et al.* (1988), a full length cDNA clone of this gene (E126) was isolated for sequence comparison and is discussed in the next Chapter.

For both the "early" and "late" stage-specific clones chosen for further analysis, a genomic sequence containing the gene of interest was isolated from a genomic library. The sequence of each gene was characterised and the 5' non-coding flanking sequences isolated. The following Chapter outlines the characterisation of each gene and describes the isolation of their promoter sequences.

CHAPTER 7 : GENE SEQUENCE CHARACTERISATION

7.1 INTRODUCTION

In the previous Chapter two cDNA clones were chosen, based on the expression patterns of the genes they represent, for isolation of their promoter sequences. The primary purpose of the work undertaken in this Chapter is the further characterisation of each gene. In particular, available literature information was used to identify sections within each sequence that may have a functional role in gene regulation, or in the case of the "late" stage-specific gene, may imply a specific function for the protein. Finally, the 5' non-coding regions of each gene was isolated from the appropriate genomic clones, ready for integration into a reporter vector.

7.2 "EARLY" STAGE-SPECIFIC SEQUENCE

7.2.1 Promoters of Ribosomal Protein Genes

There are approximately 75 yeast ribosomal proteins (rp) and four ribosomal RNAs which are thought to be produced in equimolar amounts (Raue and Planta, 1991). Whereas the equimolar production of the 17S, 5.8S and 26S rRNA species is ensured by the organisation of these genes into multiple tandem copies of a contiguous transcription unit, no such linkage exists for the genes encoding ribosomal proteins. Molecular genetic studies of yeast rp-genes have shown that they are generally scattered throughout the yeast genome (Warner *et al.*, 1986; Planta *et al.*, 1986). Despite this, and the difference in gene copy number, the cellular levels of the mRNAs are approximately equal (Kim and Warner, 1983). Since the various rp-mRNAs have about the same stability (Kim and Warner, 1983) this suggests that the rate of ribosomal proteins produced is controlled primarily at the level of transcription. Therefore, it has been hypothesised that co-ordinate transcription of these scattered genes, to ensure the production of equimolar amounts, might be mediated by structurally similar promoters of similar strength.

The first intimation of possible *cis*-acting elements involved in the transcriptional control of ribosomal protein synthesis came from the comparison of the 5' flanking regions of 21 different yeast rp-genes (Teem *et al.*, 1984; Leer *et al.*, 1985). This comparison revealed two conserved elements, at the time designated HOMOL1 (consensus AACATCC/TG/ATA/GCA) and RPG (consensus ACCCATACATT/CT/A) boxes, located 250 to 450 base pairs upstream from the ATG start codon, present mostly in tandem arrangement and in both possible orientations.

The HOMOL1 and RPG boxes, although originally considered distinct elements, were recognised by Woudt *et al.* (1986) as different representatives of the same functional sequence (now called the RPG box) since many of the nucleotides are similar for both. This led to the proposal of a more general consensus for the conserved upstream sequences of yeast ribosomal protein genes (aPyCPyPutPuCaPyPyt/a; where capitals are invariant positions and small lettering at positions with preference for the indicated nucleotide) (Woudt *et al.*, 1986). With few exceptions, yeast rp-genes carry two copies of the RPG box in either orientation (Planta and Raue, 1988).

Deletion mapping and linker-scanning experiments, carried out on several rp-genes demonstrated that these elements play a central role in controlling rp-gene expression and hence are considered to be upstream activating sequences (Rotenberg and Woolford, 1986; Woudt *et al.*, 1986; Larkin *et al.*, 1987; Schwindinger and Warner, 1987; Kraakman *et al.*, 1989). By bandshift assays and footprint analyses, these upstream sequences were identified as binding sites for the multifunctional protein RAP1 (Hoekstra *et al.*, 1994) also described by others as TUF (Vignais *et al.*, 1987; Woudt *et al.*, 1987; Hamil *et al.*, 1988).

Evidence is also accumulating that additional factors may be involved in ribosomal protein synthesis. A few yeast rp-genes have been found that do not carry any RPG boxes in their 5' flanking sequences. Footprint and methylation interference analyses of those genes without an RPG box, identified the binding site PuTCPuPyN_5ACG (Dorsman *et al.*, 1989) which was found to be necessary for the binding of a specific abundant protein originally called SUF or TAF (Vignais *et al.*, 1987; Hamil *et al.*, 1988; Dorsman *et al.*, 1989), now generally designated ABF1 (Raue and Planta, 1991). The regulatory protein ABF1 binds at approximately 160 to 200 base pairs from the ATG start codon, irrespective of the orientation of the ABF1 responsive site (Hamil *et al.*, 1988; Herruer *et al.*, 1989). ABF1 binding sites have been observed for the rp-genes encoding *L2* (Seta *et al.*, 1990), *L3* (Hamil *et al.*, 1988) and *S33* (Herruer *et al.*, 1989) and deletion analysis has shown this site to be part of the UAS of these genes (Hamil *et al.*, 1988; Herruer *et al.*, 1989).

RAP1 and ABF1 binding sites have been found, by sequence comparison, in the 5' flanking sequences of a large number of yeast genes besides those encoding the ribosomal proteins (Halfter *et al.*, 1989; Capieaux *et al.*, 1989; Seta *et al.*, 1990; reviewed by Doorenbosch *et al.*, 1992). Among these are genes encoding components of the transcriptional and translational machinery (Mann *et al.*, 1987), as well as genes involved in cellular differentiation (Hofman *et al.*, 1989), the cell cycle (Thevelein, 1991), and glycolysis (Chambers *et al.*, 1989, 1990). The common feature linking these genes is their involvement in cell growth.

7.2.2 Ribosomal Protein Gene *L45* Sequence Data

A clone containing the gene for the yeast ribosomal protein L45 was chosen for isolation of its promoter sequence. This gene was originally isolated and described by Remacha *et al.* (1988). Within the 144 "early" stage-specific clones isolated from the T1 cDNA library nine clones were identified as containing copies of the *L45* gene by colony cross-hybridisations. Amongst these clones a full length cDNA was obtained and sequenced to compare the coding and 3' non-coding sequences with those published by Remacha *et al.* (1988) (Figure 7.1). The 5' non-coding sequence of this gene was obtained by PCR amplification from the genomic clone isolated in Section 6.5. A description of the amplification procedure and sequence analysis is given in Section 7.2.3. A comparison of the amplified 5' non-coding region with Remacha's sequence is shown in Figure 7.2.

Sequencing of a complete cDNA clone revealed a 330bp open reading frame, coding for 110 amino acids. No differences were observed in the coding and 3' non-coding regions between sequence data of the cDNA clone and sequence data published by Remacha *et al.* (1988) (Figure 7.1). Comparison of the PCR generated DNA sequence to published data of the 5' non-coding region of ribosomal protein L45 (Remacha *et al*, 1988) identified two base pair differences and two insertions within the 331 amplified base pairs (Figure 7.2). The evolutionary divergence of wine yeast strains and laboratory strains, being selected for attributes in totally different environments, resulting in genomic differences such as altered ploidy levels can account for the differences found in the non-coding DNA. No changes in the sequence occur in those regions suggested to be required for transcriptional regulation by Remacha *et al.* (1988) and Mager and Planta (1990). Also, experiments that have addressed the spatial relationships between yeast promoter elements, suggest that the spacing between elements is not critical (Chen and Struhl, 1985; Hahn *et al.*, 1985).

7.2.3 PCR Amplification and Isolation of the 5' Non-Coding Sequence

As described in Section 6.5, an 18kb fragment in λgemm11 arms which hybridised to radio-labelled cDNA clone E126, was isolated from a wine yeast genomic library. Individual lambda phage isolates were prepared as described in Section 3.7.5 and used directly in a PCR DNA amplification reaction.

The primers used were specific for the 5' non-coding region of the ribosomal protein gene *L45*. The primer nucleotide sequences were determined with the computer program OLIGO 4. Each primer (RP-F and RP-R) contains a single base pair mismatch resulting in an internal RsaI restriction enzyme site.

Figure 7.1 : 950bp nucleotide sequence of the ribosomal protein gene *L45* as determined by Remacha *et al.* (1988). Includes a 460bp 5' non-coding region, a 330bp open reading frame coding for 110 amino acids, and 160bp 3' non-coding sequence.

Proposed UASs (Remacha *et al.*, 1988) starting at nucleotides -365 and -241 are underlined with an arrow indicating their orientation.

The transcription initiation start site (-81) and two potential transcription termination sites beginning at nucleotides 416 and 445 respectively are marked in blue in the 5' and 3' non-coding sequences respectively. Within the 5' flanking sequence a putative TATA box at position -125 is indicated in red.

↓designates the position of the PolyA tail addition and * marks the 5' and 3' ends of the cDNA clone isolated and sequenced within this thesis.

Figure 7.1

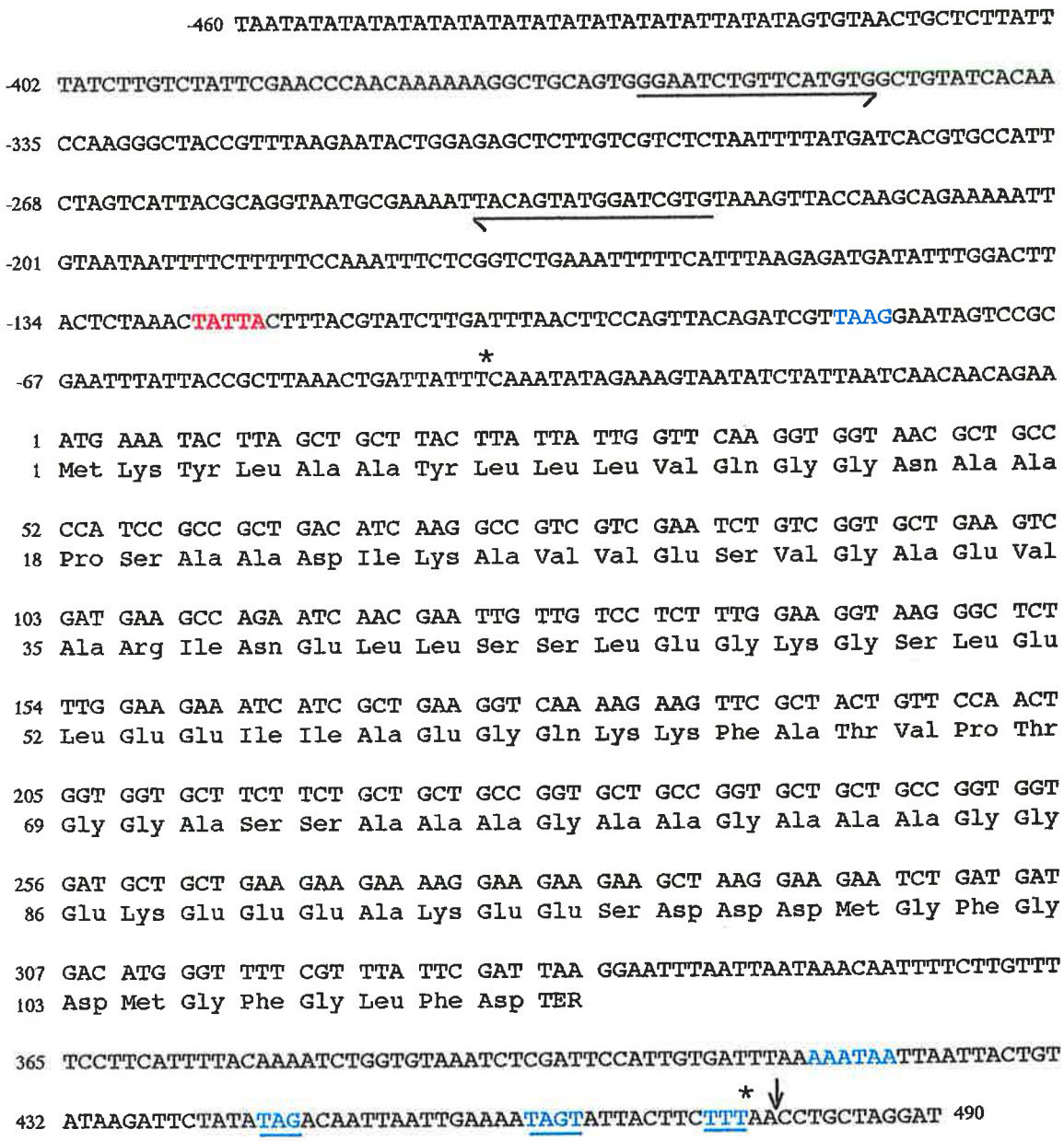

-460 TAATATATATATATATATATATATATATATATTATATAGTGTAACTGCTCTTATT
-402 TATCTTGTCTATTCGAACCCAACAAAAAAGGCTGCAGTGGGAATCTGTTCATGTGGCTGTATCACAA
-335 CCAAGGGCTACCGTTTAAGAATACTGGAGAGCTCTTGTCGTCTCTAATTTTATGATCACGTGCCATT
-268 CTAGTCATTACGCAGGTAATGCGAAAATTACAGTATGGATCGTGTAAAGTTACCAAGCAGAAAAATT
-201 GTAATAATTTTCTTTTTCCAAATTTCTCGGTCTGAAATTTTTCATTTAAGAGATGATATTTGGACTT
-134 ACTCTAAACTATTACTTTACGTATCTTGATTTAACTTCCAGTTACAGATCGTTAAGGAATAGTCCGC
-67 GAATTTATTACCGCTTAAACTGATTATTTCAAATATAGAAAGTAATATCTATTAATCAACAACAGAA
1 ATG AAA TAC TTA GCT GCT TAC TTA TTA TTG GTT CAA GGT GGT AAC GCT GCC
1 Met Lys Tyr Leu Ala Ala Tyr Leu Leu Leu Val Gln Gly Gly Asn Ala Ala
52 CCA TCC GCC GCT GAC ATC AAG GCC GTC GTC GAA TCT GTC GGT GCT GAA GTC
18 Pro Ser Ala Ala Asp Ile Lys Ala Val Val Glu Ser Val Gly Ala Glu Val
103 GAT GAA GCC AGA ATC AAC GAA TTG TTG TCC TCT TTG GAA GGT AAG GGC TCT
35 Ala Arg Ile Asn Glu Leu Leu Ser Ser Leu Glu Gly Lys Gly Ser Leu Glu
154 TTG GAA GAA ATC ATC GCT GAA GGT CAA AAG AAG TTC GCT ACT GTT CCA ACT
52 Leu Glu Glu Ile Ile Ala Glu Gly Gln Lys Lys Phe Ala Thr Val Pro Thr
205 GGT GGT GCT TCT TCT GCT GCT GCC GGT GCT GCC GGT GCT GCT GCC GGT GGT
69 Gly Gly Ala Ser Ser Ala Ala Ala Gly Ala Ala Gly Ala Ala Ala Gly Gly
256 GAT GCT GCT GAA GAA GAA AAG GAA GAA GAA GCT AAG GAA GAA TCT GAT GAT
86 Glu Lys Glu Glu Glu Ala Lys Glu Glu Ser Asp Asp Asp Met Gly Phe Gly
307 GAC ATG GGT TTT CGT TTA TTC GAT TAA GGAATTTAATTAATAAACAATTTTCTTGTTT
103 Asp Met Gly Phe Gly Leu Phe Asp TER
365 TCCTTCATTTTACAAAATCTGGTGTAAATCTCGATTCCATTGTGATTTAAAAATAATTAATTACTGT
432 ATAAGATTCTATATAGACAATTAATTGAAAATAGTATTACTTCTTTAACCTGCTAGGAT 490

Figure 7.2

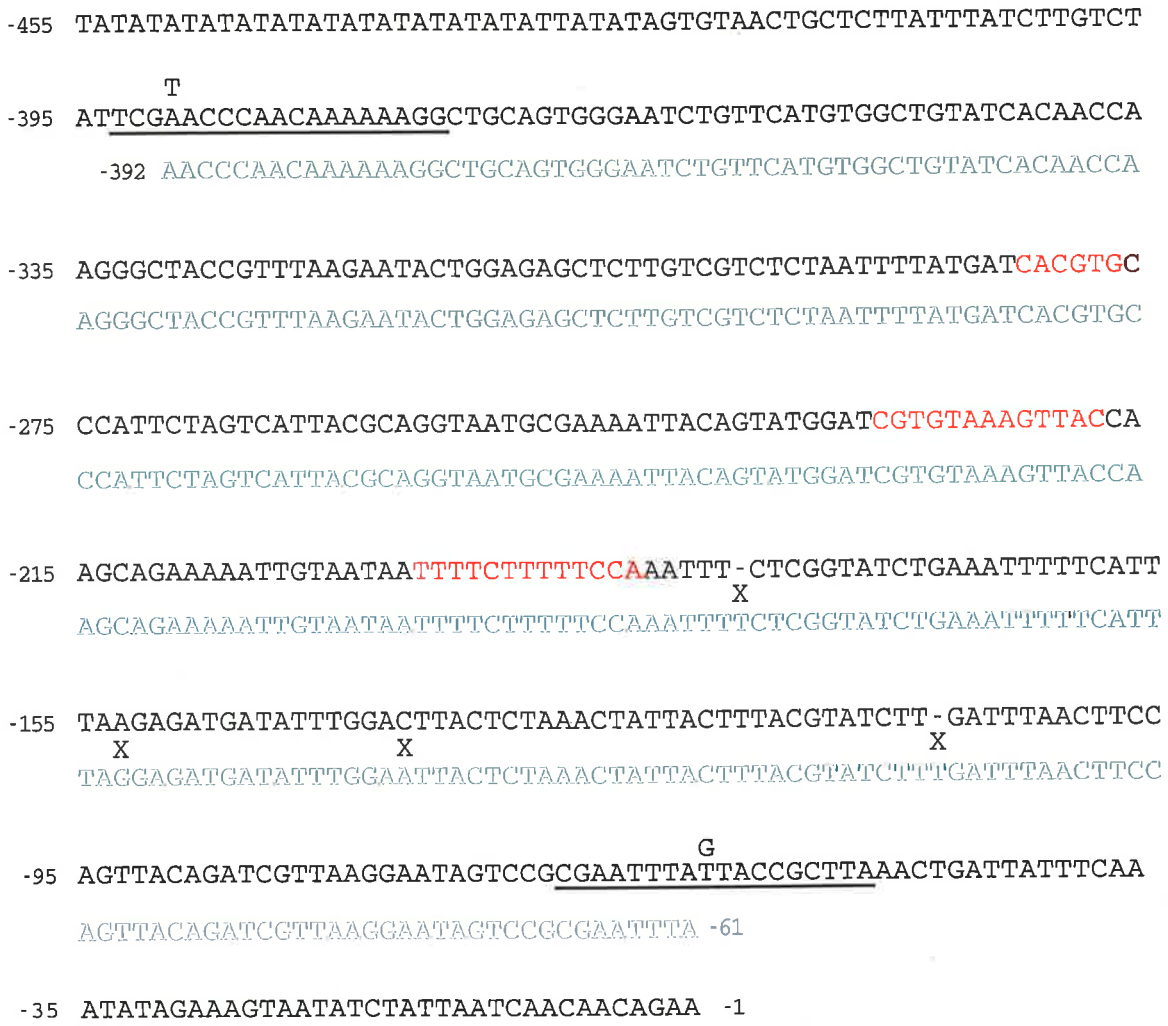


Figure 7.2 : Comparison of PCR generated DNA sequence (grey) to published data of the 5' non-coding region of ribosomal protein gene *L45* (Remacha *et al.*, 1988).

The 331bp amplified sequence contains two mismatches and two base insertions - (x).

Sequences homologous to the primers used to amplify the DNA are underlined, and the single base pair mismatches which produced functional RsaI (GTAC) restriction sites are also indicated.

Possible regulatory protein binding sites (CP1, -282; ABF1, -230; GDUF, -196) as proposed by Mager and Planta (1990) and Kraakman *et al.* (1991) are marked in red.

After amplification, size confirmation of the PCR product was obtained by visualisation of an aliquot run in an agarose gel. The DNA was ethanol precipitated, subsequently digested with RsaI and the blunt ended products isolated from a gel after electrophoresis. A proportion of the blunt ended PCR products were immediately ligated into pTZ19U to enable sequencing and confirmation that the correct fragment had been amplified. The sequence results are shown in Figure 7.2. The remainder of the blunt ended products were reserved for ligation into a reporter vector and will be discussed in the next Chapter.

7.3 "LATE" STAGE-SPECIFIC SEQUENCE

7.3.1 Isolation of the Genomic "Late" Stage-Specific Gene

As described in Section 6.5, a 16kb fragment in λgemm11 arms which hybridised to radio-labelled cDNA clone L49, was isolated from a wine yeast genomic library. Lambda DNA isolation, restriction enzyme digestion, gel electrophoresis and DNA hybridisation methods, as described in Chapter 3, were subsequently used to identify a smaller DNA sequence, within the 16kb fragment, which contained the genomic sequence of interest. This 4.0kb SacI fragment was ligated into the polylinker of vector pTZ19U. A preliminary map of restriction endonuclease sites within the 4.0kb genomic DNA subclone was constructed by conventional methods using a combination of double restriction enzyme digests of the entire insert and further subclones. This map was later refined through DNA sequencing (Figure 7.3).

An agarose gel of restriction digestions of the genomic clone, was transferred to a nylon membrane and probed with radio-labelled cDNA clone L49. This hybridisation experiment identified the position of the "late" gene within the region of the HindIII, EcoRI and KpnI restriction enzyme cluster of the 4.0kb clone, and was confirmed by sequence analysis (Figure 7.3). Figure 7.3 also demonstrates the subsequent subclones generated in plasmid pTZ19U and the sequencing strategy used to obtain a complete sequence of the "late" gene; its coding region, as well as the 5' and 3' non-coding regions.

7.3.2 "Late" Gene Sequence Data

As previously described in Chapter 5, "late" stage-specific cDNA clone L49 showed homology to a secretory glycoprotein gene (*SED1*) first characterised by Hardwick *et al.* (1992). The incomplete cDNA sequence of 144 nucleotides provided a 48 amino acid sequence which was matched via the databases, with 85 percent homology, to 48 amino acids of the total 338 amino acid sequence of the SED1 protein. This match covers a 43 amino acid repeat sequence found within the *SED1* gene (Table 7.1).

Figure 7.3 : Subcloning and sequencing strategy of the 4.0kb SacI insert of pTZ19U.

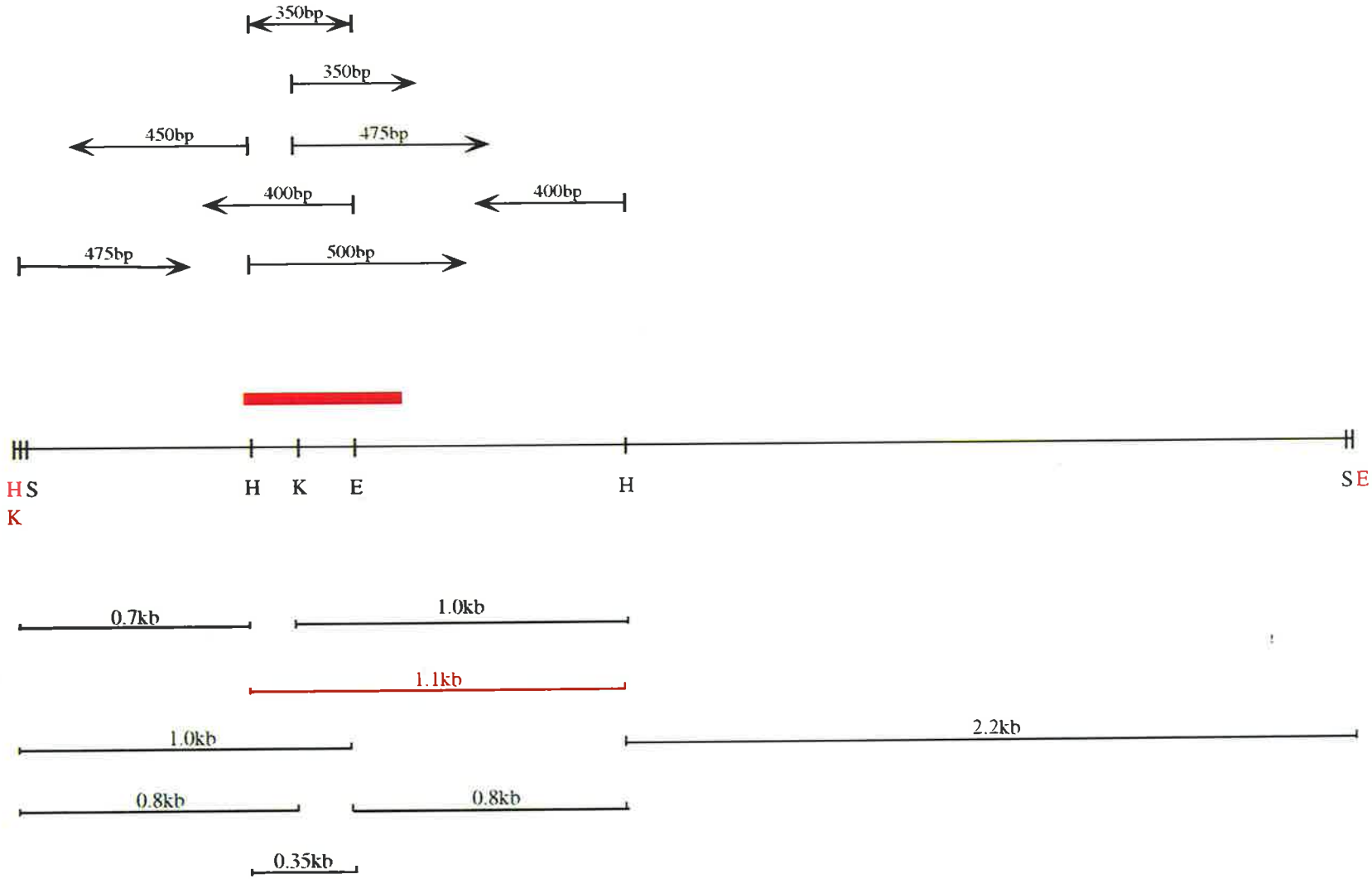
The symbol ——— represents the subclones generated and ⟶ demonstrates the direction and distance sequenced.

▬ illustrates the position of the "late" gene as determined by hybridisation analysis and confirmed by sequencing.

——— indicates the DNA subclone sequence used as a radioactive probe in Figure 7.6.

The restriction enzymes used were E - EcoR I, K - Kpn I, H - Hind III and S - Sac I. (pTZ19U polylinker sites are indicated in red)

Figure 7.3


350bp
350bp
450bp
475bp
400bp
400bp
475bp
500bp
HS
K
H
K
E
H
SE
0.7kb
1.0kb
1.1kb
1.0kb
2.2kb
0.8kb
0.8kb
0.35kb

```
SED1 Y E V V S E F T T Y C P E P T T F V T N G A T F T V T A P T T L T I T N C P C T I E K
     : · : : · : · : : : : : : : : : : · : : : x : · : : : · : : : : : : : · : : : : : : :
L49  Y T V V T E F T T Y C P E P T T F T T N G K T Y T V T E P T T L T I T D C P C T I E K
```

Table 7.1 : Alignment of a section of the deduced protein sequence of L49 cDNA and genomic clone and the SED1 amino acid repeat sequence. Perfect matches are indicated by (:); mis-matches due to a single base change by (·) and mismatches due to 2 base changes by (x).

Sequencing of the complete genomic "late" gene produced a much smaller open reading frame than expected for the *SED1* gene. Analysis of the genomic clone revealed a 444bp open reading frame, without introns, encoding 148 amino acids (Figure 7.4). The open reading frame and deduced amino acid sequence were determined by the computer program "DNA Strider".

Comparison of both the complete nucleotide sequence and the deduced primary amino acid sequence with those available from databases revealed the top three matches, each with a homology of 45.8 percent. The sequences identified were the *SED1* gene and two cosmid sequences of *S. cerevisiae* chromosomes I and IV. All include the 43 amino acid repeat sequence which was the basis for the homology observed between the deduced protein sequence of the cDNA clone and SED1. No sequences flanking the repeat showed significant homology to the "late" gene sequence. The significance of a copy of this 43 amino acid repeat within the "late" gene is unknown. However, the possibility of this gene having other similarities with the *SED1* gene and therefore other cell surface glycoproteins, was investigated and is outlined in the discussion.

Hydrophobicity of the "late" gene amino acid sequence was also analysed using the DNA program "MacMolly", with a window of 11 amino acids, and is shown in Figure 7.5. Both the amino and carboxy terminal ends are observed to be hydrophobic.

7.3.3 Mapping of the "Late" Gene to a Chromosome

In recent years, pulse field gel electrophoresis has made it possible to electrophoretically separate intact yeast chromosomes on a gel (Carle and Olson, 1985; DeJonge *et al.*, 1986). Using this technique, an experiment was also carried out to map the uncharacterised "late" gene to a chromosome.

Figure 7.4 : 840bp sequence of the "late" gene determined from a 4.0kb genomic sequence. Includes a 384bp 5' non-coding region, a 444bp open reading frame coding for 148 amino acids and a 112bp 3' non-coding sequence.

Puatative transcription initiation (-47, -36, -29, -23) and termination (504, 537) sites are marked in blue in the 5' and 3' non-coding sequences. A potential TATA box at position -115 in the 5' flanking sequence is indicated in red.

Threonine and serine residues which may act as O-glycosylation sites are marked in pink and proline rsidues needed to form β turns are indicated in green. Potential N-glycosylation sites within the open reading frame are underlined.

Figure 7.4

```
                                -384 CCATGCCGGGACTGCGGGGCCCTATCGGGGCTCGAACCCGAATCCCGCGA

-335 GTATTTATTTGAGGTCCGGGCGCAAGTTACCTAATCTGGTTTAATGATATCCCATTTAGGCGATGAC

-268 GTTCCTTCCCCTCACCCCTCGGCTGTTAGAAGATCTATTGTTATAGCCTCCTCTGGAAGAATTTATG

-201 CCAGATGAAGAAGAAAAGTTCTCGAATCCCCAGATGCCCAAATGAGGGCTTTCCATCCCTGTTAGCT

-134 GGAAAAGTGTAAGTATATCTATATAAAAATCGGCTTACTTTTGCCAGGTTCGTCTTTCACTTGCACT

 -67 CTCTTGATCTTACTTTCTACTCAAAAAGAATCAATACACAAAAATAAAATCAGTACTATTACTAATA

   1 ATG TTG TCT AAC GCT AAG CTT CTT CTA TCA TTG GCC ATG GCC TCT ACG GCT
   1 Met Leu Ser Asn Ala Lys Leu Leu Leu Ser Leu Ala Met Ala Ser Thr Ala

  52 CTC GGA TTG GTA TCT AAT TCT AGT TCC TCT GTA ATC GTG GTA CCA TCA AGC
  18 Leu Gly Leu Val Ser Asn Ser Ser Ser Ser Val Ile Val Val Pro Ser Ser

 103 GAT GCT ACT ATT GCC GGT AAC GAT ACA GCC ACG CCA GCA CCA GAG CCA TCA
  35 Asp Ala Thr Ile Ala Gly Asn Asp Thr Ala Thr Pro Ala Pro Glu Pro Ser

 154 TCC GCC GCT CCA ATA TTC TAC AAC TCG ACT GCT ACT GCA ACA CAG TAC GAG
  52 Ser Ala Ala Pro Ile Phe Tyr Asn Ser Thr Ala Thr Ala Thr Gln Tyr Glu

 205 GTT GTC AGT GAA TTC ACT ACT TAC TGC CCA GAA CCA ACG ACT TTC GTA ACG
  69 Val Val Ser Glu Phe Thr Thr Tyr Cys Pro Glu Pro Thr Thr Phe Val Thr

 256 AAT GGC GCT ACA TTC ACT GTC ACT GCC CCA ACT ACG TTA ACA ATT ACC AAC
  86 Asn Gly Ala Thr Phe Thr Val Thr Ala Pro Thr Thr Leu Thr Ile Thr Asn

 307 TGT CCT TGC ACT ATC GAG AAG CCT ACT TCA GAA ACA TCG GTT TCT TCT ACA
 103 Cys Pro Cys Thr Ile Glu Lys Pro Thr Ser Glu Thr Ser Val Ser Ser Thr

 358 CAT GAT GTG GAG ACA AAT TCT AAT GCT GCT AAC GCA AGA GCA ATC GCA GGA
 120 His Asp Val Glu Thr Asn Ser Asn Ala Ala Asn Ala Arg Ala Ile Ala Gly

 409 GCC CTA GGT TTG GCT GGT GCA GTT ATG ATG CTT TTA TGA TCGAATAGTCTTTAA
 137 Ala Leu Gly Leu Ala Gly Ala Val Met Met Leu Leu TER

 463 CTAGAGACTTATTTTAAACAGGACTATTGTATTCTTCGATGTAGTCGTTATAGTTCTTTTTCTTCAA

 530 ATCATTTAAATAAGAAACTATTTTGCC 556
```

Figure 7.5

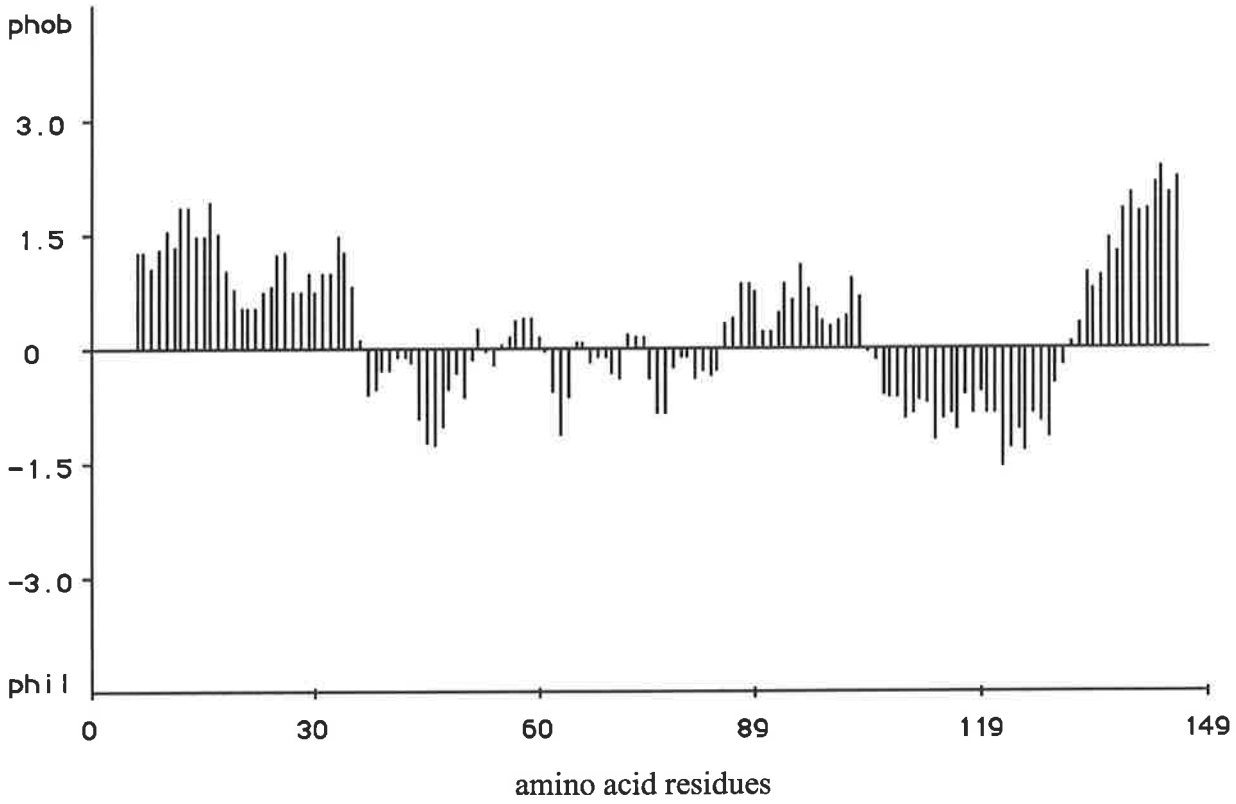


Figure 7.5 : Hydrophobicity of the "late" gene putative amino acid sequence analysed, with a window of 11 amino acids, using computer program "MacMolly".

The chromosome banding patterns of a wine yeast strain AWRI 796 and a control standard *S. cerevisiae* strain (Biorad, YNN295) were obtained and are compared in Figure 7.6a. The standard strain produced 14 bands. On the basis of data from genetic maps of *S. cerevisiae* and chromosome physical size (Mortimer and Schild, 1985; Traver and Davis, 1988), Biorad have assigned chromosome numbers and approximate sizes for each band as shown in Figure 7.6a. The largest chromosomes, being difficult to differentiate, appear as a single band, although the staining intensity of these bands implies they are doublets.

The wine yeast strain AWRI 796 revealed a banding pattern only slightly different from the standard; a single band appears in the position corresponding to two bands, representing chromosomes V and VIII, in the standard strain. However, as already indicated, the number of bands does not necessarily correlate with the number of chromosomes. The broadness and intensity of this single band would imply that these chromosomes, for this strain, were not resolved under these conditions.

A nylon membrane of this gel was prepared by the method of Southern (1975) and probed with a radio-labelled HindIII fragment of the "late" genomic clone (indicated in Figure 7.3). The filter was placed next to autoradiographic film and developed after 48 hours exposure (Figure 7.6b). A single strong radioactive band appears in each lane. Wine yeast strain AWRI 796 hybridises with the doublet containing chromosomes V and VIII. However, these bands are resolved in the standard strain and hybridisation only occurs with the larger band (chromosome V) in this lane. This result suggests that the uncharacterised "late" gene is located on chromosome V.

7.3.4 Isolation of the "Late" Gene 5' Non-Coding Region

Once the complete sequence of the "late" gene and its flanking non-coding regions were determined, the 5' end was searched for appropriate restriction enzyme sites in which to isolate the putative promoter sequence. Figure 7.7 indicates the sequence isolated and the restriction enzyme sites used to obtain this fragment.

A subclone, containing the desired 5' non-coding sequence, isolated during the sequencing strategy was digested with HindIII and EcoRV. After ethanol precipitation the DNA was end filled and the resultant blunt ended fragment isolated from an agarose gel. The blunt ended fragments were ligated into pTZ19U for sequencing and confirmation that the correct sequence had been isolated. After confirmation, the remainder of the blunt ended fragments were then reserved for ligation into a reporter vector which will be discussed in the following Chapter.

Figure 7.6

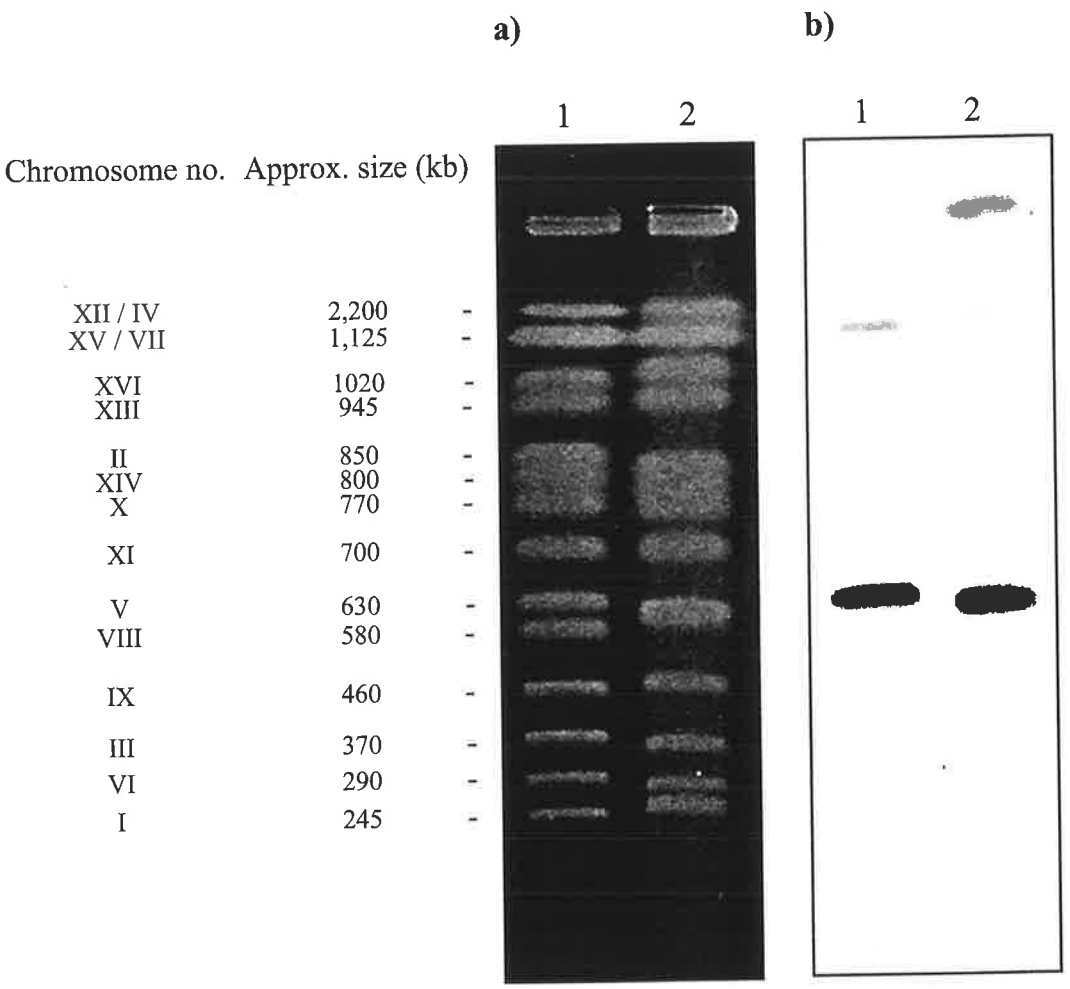


Figure 7.6 : Hybridisation depicting the chromosomal location of the "late" gene.

a) Yeast chromosomes, of strains 1 - YNN295 and 2 - wine yeast strain AWRI 796, separated by pulse field gel electrophoresis.

b) Southern transfer of chromosome patterns depicted in panel a) probed with radio-labelled HindIII sequence of the isolated genomic clone (indicated in Figure 7.3).

Figure 7.7

Figure 7.7 : 5' non-coding region of the "late" genomic clone isolated for insertion into a reporter vector.

The "late" gene coding sequence is indicated in italics.

Restriction enzyme sites used for the isolation of the sequence are double underlined; EcoRV (GATATC) and HindIII (AAGCTT).

Matches to the heat shock element consensus sequence are indicated in red with nucleotides matching the exact consensus underlined. Nucleotides that are not part of a larger consensus but match individual HSE subcomponents (GAA or CTT) are also underlined.

7.5 DISCUSSION

A clone containing the gene for the yeast ribosomal protein L45 was chosen for isolation of its promoter sequence. This gene was originally isolated and described by Remacha *et al.* (1988). Further characterisation of the 5' flanking sequences of *L45* has been undertaken by Mager and Planta (1990) and Kraakman *et al.* (1991) during their analysis of activation sites of yeast ribosomal protein genes.

The open reading frame of the L45 ribosomal protein gene codes for 330 amino acids. Bennetzen and Hall (1982b) identified codon preference in some yeast genes and correlated high levels of gene expression with codon bias. Analysis of the *L45* gene open reading frame shows that it is largely (90%) composed of codons which are preferentially chosen, suggesting that L45 is a highly expressed protein.

The 3' untranslated region of the cDNA extends approximately 150bp from the TGA stop codon to the polyA tail. Within this region, at position 416, there is an AAATAA sequence proposed by Remacha *et al.* (1988) as the transcription termination site. This hexanucleotide, or a closely related sequence, is generally found 10 to 30 base pairs upstream of the polyadenylation site in a majority of eukaryotic mRNAs (Proudfoot and Brownlee, 1974) and is absolutely required for polyadenylation of the mRNA (Fitzgerald and Shenk, 1981: Wickens and Stephenson, 1984: Zarkower *et al.*, 1986). However, Zaret and Sherman (1982) suggest that yeast, unlike higher eukaryotes, do not use the AAUAAA mRNA 3' end processing signal. From a comparison of yeast genes, Zaret and Sherman (1982) have proposed a consensus TAG...TA(T)GT...TTT (where the sequences between the blocks is variable) as a sequence that signals transcription termination at a short distance downstream. Sequences homologous to Zaret and Sherman's consensus can also be observed in the 3' end of *L45*, extending from position 445 for over 33bp. Both putative transcription termination sequences are identified in Figure 7.1.

Neither proposed transcription termination site matches the consensus sequences or spatial requirements perfectly. The higher eukaryote consensus sequence is found nearly 100bp from the polyadenylation site, a distance suggested by the literature to be excessive. However, Zaret and Sherman's (1982) triparite sequence, although closer to the polyadenylation site, spans more bases than is often seen in yeast genes. Deletion analysis and the observation of run-through transcripts would help determine the individual sequences of this gene required for efficient transcription termination.

Possible sequences for the transcription initiation site consist of a PyAAPu consensus sequence which is common to other yeast genes (Dobson *et al.*, 1982; Burke *et al.*, 1983) many of which may be between the putative TATA and the first codon. However, by S1 nuclease mapping, Remacha *et al.* (1988) identified the TAAG sequence at -80 as being the transcription initiation site (Figure 7.1). Sequence analysis of the region upstream from the transcription initiation site and downstream from possible transcription activation sites (to be discussed) identified only a single putative TATA element, situated at -125, with a sequence of TATTA (Figure 7.1).

Remacha *et al.* (1988) attempted to find the RPG consensus sequence in the promoter region of ribosomal protein L45. They found an RPG box on both strands, in opposite orientation over 100 nucleotides apart (Figure 7.1). However, the quality of fit for these sequences is poor and no deletion experiments were undertaken to determine if these sequences were absolutely required for transcription. Later, Mager and Planta (1990) and Kraakman *et al.* (1991) suggested a completely different range of upstream activating sequences for the promoter of *L45*. By band shift experiments they identified three possible regulatory protein binding sites (CP1, ABF1 and GDUF) contained within 375bp upstream of the translational start site. Deletion experiments confirmed the ABF1 and GDUF protein binding sites as regulatory elements of this gene. A severe decline in L45 mRNA was detected with deletion of the ABF1 site. The residual transcription observed after deleting the ABF1 site was further removed by the deletion of the GDUF site (Mager and Planta, 1990; Kraakman *et al.*, 1991). These sequences are identified in Figure 7.2.

The 5' flanking region of the *L45* gene containing all the key sequences for regulation, as described above, was isolated using PCR amplification for use as potential promoter sequences. A description of its use in a reporter vector is outlined in the next Chapter.

The DNA sequence of a genomic clone containing the "late" gene was determined. Within this sequence only one sizeable open reading frame encoding a polypeptide of 148 amino acids was found. Analysis of this putative coding sequence revealed a codon bias of 58 percent indicating a relatively abundant protein (Bennetzen and Hall, 1982b). Comparison of both the "late" gene nucleotide sequence and deduced primary amino acid sequence with those available on the data bases revealed similarities with a 43 amino acid repeat sequence found in the *SED1* gene and two cosmid sequences of chromosomes I and IV of *S. cerevisiae* (Table 7.1). The *SED1* gene has been characterised by Hardwick *et al.* (1992) as a secretory glycoprotein. The significance of a copy of this 43 amino acid repeat within the "late" gene is unknown. However, the possibility of this gene having other similarities with the *SED1* gene and therefore other cell surface glycoproteins, was investigated.

Much of the SED1 amino acid sequence is repetitive; besides the perfect 43 amino acid duplication, there are many short repeats. These repeat features are characteristic of a number of O-glycosylated, secreted or GPI-anchored cell surface glycoproteins in *S. cerevisiae*; for example *AGA1* (Roy *et al.*, 1991), *KRE1* (Boone, *et al.*, 1990b) and *HSP150* (Russo *et al.*, 1991a) gene products. Analysis of the "late" gene putative protein sequence however, did not reveal a duplication of the 43 amino acid sequence or any other significant repeat sequences.

Glycosylated proteins undergo extensive carbohydrate modifications after translation and a number of features which are required for these modifications have been identified. Protein glycosylation in yeast involves the attachment of saccharide moieties to the protein either via an asparagine amide (N-linked) or via a ser/thr hydroxyl (O-linked) linkage. Therefore, another feature of glycosylated cell surface proteins is their richness in serine and threonine residues (Frevert and Ballou, 1985; Tanner and Lehle, 1987). Not only are these residues potential sites for O-glycosylation they may also play a role in hydrogen bonding to cell wall carbohydrates (Roy *et al.*, 1991). Examples of ser/thr richness in glycoproteins range from 25 percent with the secretory glycoprotein HSP150 (Russo *et al.*, 1991a) to 50 percent with GPI-anchored cell surface glycoprotein AGA1 (Roy *et al.*, 1991). Examination of residues in the "late" gene identified 30 percent of amino acids as being either serine or threonine, although no ser/thr residues were found in the last 23 amino acids at the carboxy terminus (Figure 7.4).

No consensus sequence for O-glycosylation has been reported. However, O-glycosylated serine and threonine amino acids frequently have nearby proline residues needed to form β turns (Lehle and Bause, 1984; Gellerfors *et al.*, 1989). On the basis of this characteristic it is postulated that a critical feature to determine whether or not serine and threonine residues become O-glycosylated is exposure on the surface of the protein (Lehle and Bause, 1984; Tanner and Lehle, 1987). Within the "late" gene amino acid sequence there are 10 proline residues present, 6 of which are placed directly next to a ser/thr residue with the remaining 4 no more than 3 amino acids away from a serine or threonine. Again no proline amino acids are found in the last 23 amino acids at the carboxy terminus (Figure 7.4).

Besides the potential O-glycosylation sites 3 potential N-glycosylation sites were also found within the "late" gene amino acid sequence and are marked in Figure 7.4. The requirements for N-glycosylation is the tripeptide sequence asn-x-ser/thr where x is not proline (Lehle and Bause, 1984; Tanner and Lehle, 1987). This conserved signal is necessary but not always sufficient to determine whether N-linked glycosylation will

occur. Consequently, without analysis of the protein product it is impossible to say conclusively that the "late" gene protein is either O- or N- glycosylated. However, O-glycosylation involves the attachment of short mannose-oligosaccharides linked through the hydroxyl groups of serine and threonine to the protein. Hence, confirmation of glycosylation could be monitored by the incorporation of tritiated (^3H) mannose. Such an experiment was undertaken by Russo *et al.* (1991a) to confirm glycosylation of a secreted heat shock protein. Further information on protein glycosylation in yeast is available in reviews by Tanner and Lehle (1987), Ballou (1990), as well as Herscovics and Orleans (1993).

Glycoproteins are mainly found among extracellular, vacuolar and membrane bound enzymes, among cell wall structural components and sexual agglutinins. Most remain intercollated in the cell wall and a few may be secreted across the cell wall into the growth medium (Tanner and Lehle, 1987). Such proteins contain signal sequences at their amino terminal ends that are responsible for their proper localisation within the cell or to enable their secretion.

Signal peptides have a low degree of sequence conservation and only possess an overall design in common. Typically, their length is between 15 and 30 residues and they show three distinct regions; a positively charged N-terminal n-region, a hydrophobic h-region and a more polar C-terminal c-region (von Heijne, 1988; Briggs and Gierasch, 1986). The n-region varies in length and nature of amino acids but always has a positive charge. The h-region possesses hydrophobic residues (mainly leu, ala, met, val, ile, phe and trp but also occasionally pro, gly, ser and thr) linked together in what is essentially a random order. This hydrophobic core is the true hallmark of signal sequences (Gierasch, 1989; Heslot and Gaillardin, 1992b). The c-region has a more specific pattern with respect to the cleavage site and obeys the "-3, -1" rule: the residues at -1 and -3 must be small and uncharged. In contrast, bulky and charged residues are usually present at position -2 (von Heijne, 1983; von Heijne, 1984).

Hydrophobicity of the amino acid sequence was analysed, with a window of 11 amino acid residues, and is shown in Figure 7.5. Both the amino and carboxy terminal ends are observed to be hydrophobic. A hydrophobic amino terminal supports the idea of a signal sequence and indeed a core sequence of approximately 12 amino acids, starting from residue 7, comprises of amino acid residues consistent to the h-region. However, terminal to this region many amino acids are present that match the "-3,-1" rule, subsequently the determination of a specific cleavage site is ambiguous.

Hydrophobicity was also observed in the carboxy terminus. Proteins that are localised to the cell surface are produced with a hydrophobic carboxy termini which has been shown to serve as a membrane spanning domain or to provide a signal for the attachment of a glycosyl-phosphatidylinositol (GPI) membrane anchor (Ferguson and Williams, 1988; Low and Saltiel, 1988; Conzelmann *et al.*, 1988). The glycoproteins SED1, AGA1 and KRE1 all have hydrophobic C-terminal sequences also lacking in ser/thr residues despite their ser/thr richness. A similar situation is observed in the "late" gene. Therefore, the hydrophobic carboxy terminus of the "late" gene could potentially act to direct the attachment of a GPI anchor.

The amino and carboxy terminal amino acid sequences are hydrophobic, suggesting the presence of signal sequences that direct the protein to its cellular localisation and possibly aid in its attachment to the cell wall. In both events the hydrophobic signal sequences would be cleaved from the protein. Therefore, isolation of the protein product and direct amino acid sequencing could determine the cleavage sites and the sequence removed.

In conclusion, many features observed within the "late" gene open reading frame have similarities to those found in other cell surface glycoproteins. Therefore, by analogy, the "late" gene may also be localised at the yeast cell surface. Further information on cell wall assembly in yeast has been compiled in reviews by Fleet (1985, 1991) and Klis (1994).

Analysis was then undertaken of the 5' and 3' non-coding regions of the "late" gene for possible identification of signal sequences involved in transcriptional control. Examination of the 3' flanking region identified a putative yeast mRNA transcription termination signal which conforms perfectly to Zaret and Sherman's (1982) triparite signal sequence, starting at nucleotide 504 and stretching for the next 20 bases. Alternatively, the sequence AAATAA at 537, which is a modification of the eukaryotic transcription termination consensus, is also present (Figure 7.4). As only an incomplete "late" gene cDNA clone was isolated, there is no indication of the position in which the poly(A) tail is attached. Deletion analysis and the observation of run-through transcripts is required to determine the individual sequences of this gene required for efficient transcription termination.

Sequence analysis of the region upstream from the translation initiation codon revealed only a single putative TATA box (matching perfectly with the consensus sequence TATAAA) starting at position -117 (Figure 7.4). Using the consensus sequence PyAAPu for the transcription initiation start site (Dobson *et al.*, 1982; Burke *et al.*, 1983), many putative sequences (at positions -47, -36, -29 and -23) can be identified between the TATA box and the first translatable codon (known as the initiation window) (Figure 7.4). As the actual start sites in this window are probably selected by a mechanism that prefers

sequences proximal to the TATA element (Mellor, 1993) it is likely that the site at position -47 is the transcription initiation site. Confirmation of this supposition would require analysis of the 5' end of a full length cDNA clone. Also observed in the 5' non-coding sequences, is the universal preference for an adenine residue at positions -3 and -1 first described by Hamilton *et al.* (1987).

Another interesting feature of the 5' non-coding region is the prevalence of the triplet sequence GAA/TTC. Inspection of a large number of heat shock regulatory elements (HSE) revealed that the periodic arrangement of GAA blocks is a common feature (Pelham, 1985). A heat shock regulatory consensus sequence has been defined as three or more contiguous repeats of a five nucleotide element, NGAAN, in altering orientations (Amin *et al.*, 1988), although in several instances the regions of heat shock elements appear to consist of interrupted arrangements of the GAA/TTC blocks (Amin *et al.*, 1988; Xiao and Lis, 1988; Susek and Lindquist, 1990; Russo *et al.*, 1991a). Susek and Lindquist (1990) identified 5 elements, by deletion analysis, necessary for transcription of *HSP26* all of which were variations on the HSE consensus sequence and none of which contained all 3 elements. Analysis of the 5' non-coding region of the 'late" gene identified a series of heat shock like regulatory elements. These elements are indicated in Figure 7.7.

The heat shock response has also been shown to occur under a wide variety of conditions. For example, the heat shock protein HSP26 is induced by heat shock, stationary phase arrest or nitrogen starvation (sporulation conditions) (Kurtz and Lindquist, 1984; Kurtz *et al.*, 1986; Susek and Lindquist 1990). Susek and Lindquist (1990) presented several lines of evidence to suggest that a common regulatory mechanism is employed under all induction conditions. It is possible that such regulatory factors also have an effect here but, without functional tests their significance is unclear. Most of the HSEs in the "late" gene upstream sequence are very weak matches to the consensus and would not be expected to be functional on their own. However, they may work cooperatively or in conjunction with other elements. To better elucidate the mechanism of "late" gene regulation, deletion analysis of this region and RNA hybridisation experiments need to be carried out to identify the specific *cis*-regulatory sequences.

Further characterisation and functional investigation of the "late" gene could include a single step gene disruption experiment, as described by Rothstein (1983), to assess whether the "late" gene is essential to cell survival. Another informative experiment may be the overexpression of the "late" gene and an observation of its effect on cell growth and viability. If the protein is associated with the cell wall both experiments may cause a reduction in the cell wall or a disruption to the cell wall ultra-structure. Electron micrographs may be used to assess any effect. Such experiments have been conducted by

Boone *et al.* (1990b) in the analysis of the *KRE* gene found to be associated with cell wall β-glucan assembly. Alternatively, it may be possible to assess an effect of cell wall alteration by analysis of the cell growth and viability under the extreme fermentation conditions in which this sequence was selected.

Given the information provided in the literature, either specific for the ribosomal protein L45 or the more general information on promoter structure, as described in the literature review, both 5' non-coding sequences isolated within this Chapter were considered to contain the necessary sequences to regulate gene expression. The following Chapter describes the introduction of these sequences into a repoter vector to monitor their control on gene expression.

CHAPTER 8 : FUNCTIONAL ANALYSIS OF PUTATIVE PROMOTER SEQUENCES

8.1 INTRODUCTION

The study of regulatory elements has in the past posed unique problems for the investigator. Promoters of genes that are critical to the cell, or toxic when over produced, or whose products cannot be easily measured, were difficult to study. However, a gene fusion can be constructed between the promoter of interest and a reporter gene, that is, a gene encoding an easily assayable protein that can confer a detectable phenotype on the host. The promoter can then be analysed independently of the gene it controls. In this way one can focus on the effects of alterations in the regulatory sequence without affecting the host cells functions.

A procedure is described here which employs the *E. coli* β-glucuronidase (*uidA*) gene as a reporter for expression analysis in order to determine the ability of isolated sequences to act as functional promoters.

8.2 VECTORS FOR THE ANALYSIS OF REGULATORY SEQUENCES

A description of vector components required for efficient yeast transformation is provided in the literature review. In addition to such sequences necessary for the selection and replication of the plasmid within a yeast host, specific requirements are also needed for reporter vectors. These include, most importantly, a reporter gene. Commonly used reporter genes used in yeast include the *E. coli* derived genes *lacZ* (Rose *et al.*, 1981; Guarente and Ptashne, 1981; Casadaban *et al., 1983;* Ruby *et al.*, 1983; Santangelo *et al.*, 1986, 1988), *CAT*, an antibiotic resistance gene encoding the protein chloramphenicol acetyltransferase (Cohen *et al.*, 1980; Prost and Moore, 1986; Achen *et al.*, 1986), and *GALK*, the galactokinase gene (Rymond *et al.*, 1983; Goodey *et al.*, 1986). In addition to these *E. coli* derived genes, yeast sequences such as *CYC1* (Lowry *et al.*, 1983) and *SUC2* (Emr *et al.*, 1983) have also been used as reporter genes in the analysis of regulatory sequences. More recently, a reporter sequence, the *E. coli* β-glucuronidase gene (*uidA*), used initially in the study of regulatory expression of nematodes and plants (Jefferson *et al.*, 1986, 1987; Jefferson, 1987), has been used as a novel marker gene in yeast (Petering *et al.*, 1991c). An advantage of the GUS system as a marker in yeasts strains include its low background levels and ease of assay (Petering *et al.*, 1991c).

Reporter vectors are designed because of the *cis*-acting nature of promoters. Therefore, the reporter gene must be appropriately positioned between the transcription promoter and termination elements in a vector. Consequently, upstream of the reporter there must be convenient restriction sites for the insertion of the putative promoter sequence to be tested. There are no stringent requirements for the sequence joining the promoter to the structural gene. Proper transcription termination and/or polyadenylation sequences are also required downstream of the reporter to ensure optimal mRNA accumulation. Incorrect termination will lead to low mRNA levels as overlong transcripts are unstable (Zaret and Sherman, 1982).

Once cloned, a promoter sequence requires further characterisation and the ability of a sequence to act as a functional promoter must be verified. In general, when studying the promoter region of a given gene the initial experiments are designed to determine the minimum continuous sequence necessary for wildtype levels of transcription This is accomplished by creating a series of deletions that remove DNA sequences adjacent to the 5' end of mRNA coding region. The most deleted derivative that confers normal levels of transcription contains the entire promoter region.

8.2.1 Construction of a Reporter Vector

Many reporter vectors are available commercially. However, their design was developed for transformation of haploid yeast strains and they are unsuitable for work with polyploid yeast strains. Therefore, for efficient transformation and subsequent analysis of putative promoter sequences within wine yeast, construction of a reporter vector was required.

A vector (pAW220) was developed for the transformation of wine yeast by Petering *et al.* (1991a/b). This *E. coli* / yeast shuttle vector contains an ampicillin resistance gene for selection in bacteria, a yeast dominant selectable marker *SMR1-410*, which also acts as a homologous sequence for integration, an expression cassette (containing both *ADH1* promoter and terminator sequences) and a reporter gene (β-glucuronidase, GUS) driven by the cassette (Figure 8.1). The *SMR1-410* gene on the plasmid is almost identical in sequence to the yeast *ILV2* gene; a single base mutation leads to resistance to the herbicide sulfometuron methyl (Casey *et al.*, 1988). Therefore, sufficient homology exists between the two sequences to target integration to the *ILV2* gene via homologous recombination with *SMR1-410.*

The vector (pAW220) designed by Petering *et al.* (1991a/b) was constructed to enable wine yeast strains to be tagged, providing the means for their unequivocal identification and monitoring during fermentations. As an example, the tagged strain was used to monitor killer activity of *Saccharomyces* in mixed culture wine ferments. Consequently, easily detectable GUS cell suspension and plate assays were developed for this system (Petering *et al.*, 1991a/c).

All components needed for a reporter vector of a polyploid yeast strain are present within this pAW220 construct. However, as the reporter gene is already driven by the *ADH1* promoter, manipulation of this vector was required for it to become functional for promoter analysis. This required the removal of the *ADH1* promoter and the integration of a novel cloning site which would enable ligation of other possible promoter sequences to this area. The following cloning strategy is outlined in Figure 8.1.

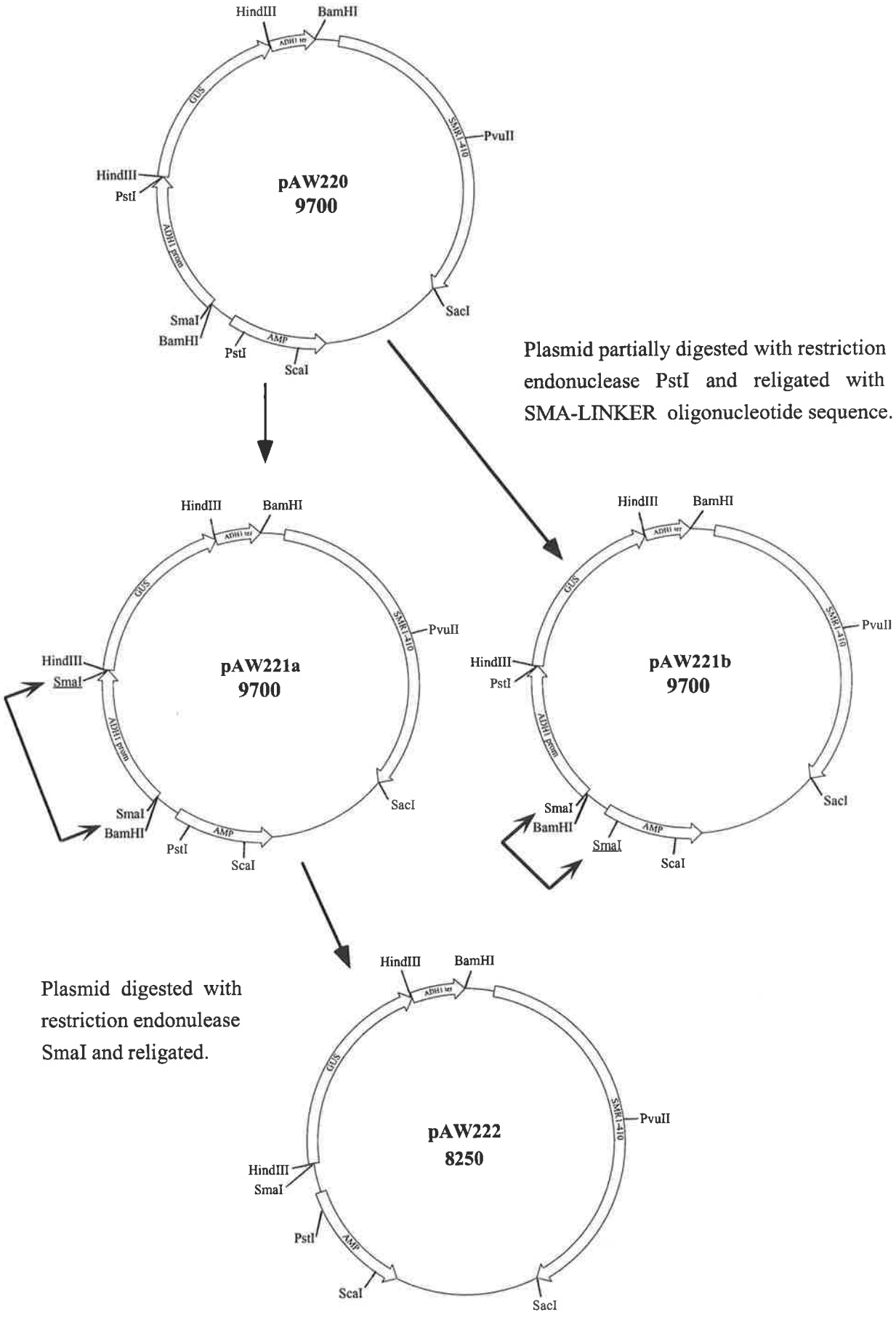
As the expression construct (promoter, terminator and reporter gene) was ligated as a unit into the vector pAW220 (Petering *et al.*, 1991a/b), no specific restriction sites were available for the removal of the *ADH1* promoter. However, a SmaI and PstI site were shown to flank either side of the promoter sequence.

Initially, the plasmid pAW220 was partially digested with the restriction enzyme PstI. There are two PstI sites within this plasmid, one within the *AMP* gene and the other flanking the 3' end of the *ADH1* promoter. Linearised plasmids were isolated and subsequently ligated with a synthetic oligonucleotide sequence (SMA-LINKER) designed to anneal to the PstI sticky ends. If annealed, religation with the oligonucleotide sequences will create a new SmaI restriction site within the PstI site. Religated plasmids were then transformed into competent *E. coli* cells, transformants were selected on ampicillin plates and plasmid preparations made. These DNA preparations were then digested with restriction endonuclease SmaI. The plasmid pAW220 already has a SmaI site, positioned adjacent to the 5' end of the *ADH1* promoter, and as a result produces a linear molecule. Hence, plasmids having integrated the synthetic olionucleotide at either PstI site will produce two bands when digested with SmaI (pAW221a/b). Of particular interest are those plasmids which have the newly created SmaI site, in the PstI restriction site, flanking the 3' end of the promoter. This will enable the removal of the *ADH1* promoter and the creation of a novel SmaI site.

After electrophoresis of the digests in an agarose gel, two banding patterns were observed. Each digestion contained a large fragment representing most of the vector and either a 1.45kb or 0.75kb fragment representing sequences flanking either side of the original SmaI restriction endonuclease site. The 1.45kb fragment contains the *ADH1* promoter

Figure 8.1 : Manipulation of the plasmid pAW220 (Petering *et al.*, 1991a/b) for the development of a reporter vector.

Figure 8.1


HindIII
BamHI
ADH1 ter
GUS
SMR1-410
PvuII
HindIII
PstI
pAW220
9700
ADH1 prom
SmaI
BamHI
AMP
PstI
ScaI
SacI
Plasmid partially digested with restriction endonuclease PstI and religated with SMA-LINKER oligonucleotide sequence.
pAW221a
9700
pAW221b
9700
Plasmid digested with restriction endonulease SmaI and religated.
pAW222
8250

while the 0.75kb fragment contains vector sequence as well as part of the ampicillin resistance gene. A plasmid producing each banding pattern is demonstrated in Figure 8.1 and are designated pAW221a and pAW221b respectively.

A DNA preparation of the plasmid pAW221a was chosen for further ligation. This preparation was digested with SmaI, ethanol precipitated and then religated. Plasmids were transformed into competent *E. coli* cells and transformants selected on ampicillin. Plasmid preparations were made, digested with restriction endonuclease SmaI and run on an agarose gel. A plasmid preparation missing the SmaI insert and hence the *ADH1* promoter, was chosen for further work (pAW222).

Experiments to isolate vectors with "early" and "late" promoter sequences in both orientations (sense and nonsense) with respect to the GUS coding region were then conducted. Potential promoter sequences were prepared for ligation, as described in the previous Chapter (Sections 7.2.3 and 7.3.4). Both "early" and "late" sequences were isolated as blunt ended fragments which were ligated into linearised SmaI digested pAW222 plasmid. Parallel ligations of sequences into SmaI digested pTZ19U were also undertaken. This enabled each fragment to be sequenced. These results are set out in the previous Chapter.

Ligated plasmids were transformed into competent *E. coli* cells and transformants selected on ampicillin. Prior to transformation, each ligation mix was digested with the restriction endonuclease SmaI. Since incorporation of the blunt ended fragments in the SmaI site of the vector will result in the disruption of the CCCGGG SmaI restriction site, digestion with SmaI before transformation will linearise the native plasmids ensuring transformation of only recombinant vectors. Plasmid preparations were made of transformants and digestion with restriction enzymes used to determine the presence of the putative promoter fragments and their orientation. The sequence data of each putative promoter insert (obtained via sequencing of the pTZ19U transformants) was used to determine single restriction sites appropriate for orientation analysis. The restriction site SacI was found in the "early" putative promoter sequence and ScaI in the "late" putative promoter sequence. Both these sites are also present, in single copy, within the vector pAW222. Figure 8.2 gives a basic graphic representation of these sites.

Subsequently, "early" (E) and "late" (L) putative promoter sequences in sense (+) and non-sense (-) orientation, ligated into the SmaI site at the 5' end of the GUS gene of vector pAW222, were prepared for transformation of wine yeast. These plasmids were designated pAW222E+, pAW222E-, pAW222L+ and pAW222L-.

Figure 8.2

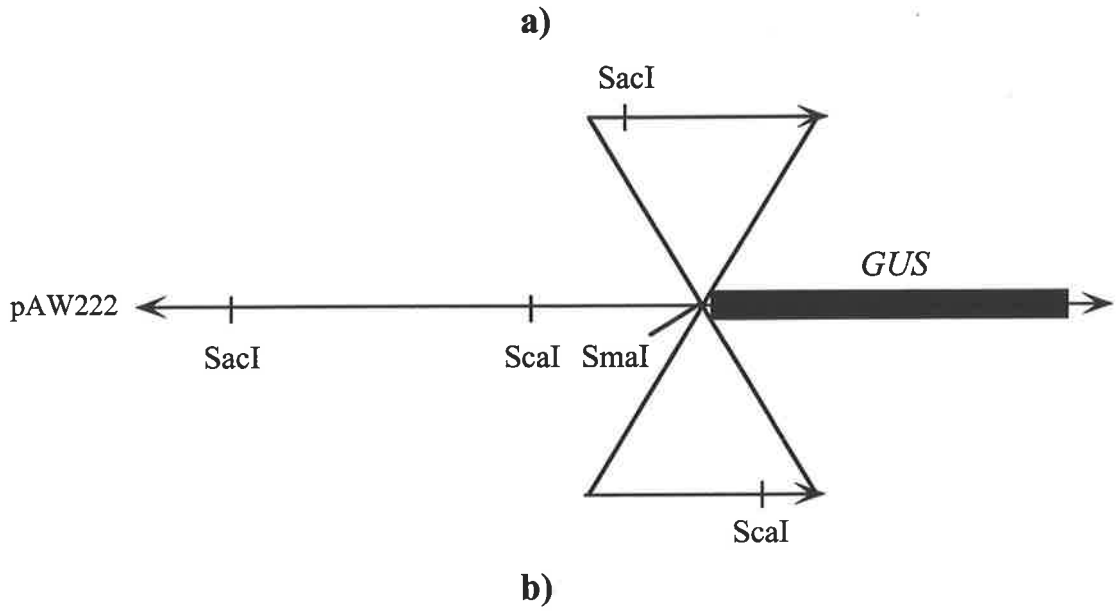


Figure 8.2 : Orientation of "early" and "late" putative promoter sequences within the GUS reporter vector.

Putative promoter sequences (E or L) were ligated into the SmaI site of the GUS reporter vector pAW222 and their orientation, sense (+) or nonsense (-), confirmed by restriction enzyme digests.

a) "early" - pAW222E+/- digested with SacI

b) "late" - pAW222L+/- digested with ScaI

Putative promoter sequences are presented above, with respect to restriction sites, in sense orientation. Diagram is not to scale.

8.3 TRANSFORMATION

Prior to transformation plasmids were digested with the restriction endonuclease PvuII. This gives rise to a linear molecule with *SMR1-410* sequences at both ends. The DNA ends are highly recombinogenic and integration is most likely to occur at the PvuII site in the *ILV2* gene. The result of this event will be two *ILV2* genes flanking the GUS cassette, one of which will contain the *SMR1-410* mutation conferring resistance to sulfometuron methyl.

PvuII digested plasmids, pAW222E+/- and pAW222L+/- and pAW220, were introduced into the wine yeast strain AWRI 796. Transformants were selected for resistance to the herbicide sulfometuron methyl (at 10μg/ml). A transformation efficiency of 7 transformants per microgram of DNA was observed. Transformed colonies were tested for GUS expression via a plate assay.

8.3.1 GUS Plate Assays

There are several sensitive assays for the β-glucuronidase enzyme that utilise commercially available substrates (Jefferson, 1987). These include substrates for spectrophotomeric, fluorometric and histochemical analyses. Petering (1991a) undertook an empirical investigation to determine the appropriate conditions for the detection of GUS activity in transformed yeast strains. The commercially available substrate used in these procedures for GUS detection is X-GLUC (5-bromo-4-chloro-3-indol β-glucuronide). β-glucuronidase cleaves the substrate X-GLUC to produce an indoxyl derivative which, upon oxidation, gives rise to an insoluble and highly coloured indigo dye. As a result, GUS activity is detected by the presence of a blue precipitate.

Transformed wine yeast colonies of each vector type were picked and streaked for single colonies on selective media (SD plus 10μg/ml sulfometuron methyl). Single colonies were then transferred to a selective media master plate and replica plated to YPD media containing X-GLUC (50μg/ml). Plate assays were conducted as described in Chapter 3, Section 3.18.1 and the presence of β-glucuronidase scored.

Transformed colonies were observed to vary, in respect to blue colour, for each plasmid type. Variation, to a lesser degree, was also observed among different transformants of the same plasmid type. Transformants containing the GUS gene driven by the *ADH1* promoter appear dark blue, those driven by "early" and "late" promoter sequences in sense orientation vary moderately in blue intensity and transformants with sequences in nonsense orientation range from light blue to no colour. Sectoring of some colonies was also observed.

A transformed colony of each plasmid type was picked and screened for the presence of the GUS construct and to verify the integration site using a DNA hybridisation method.

8.3.2 Verification of Transformation and Site-Specific Integration.

An example of each transformant type, plus an untransfomed control, were grown overnight in non-selective conditions and genomic DNA preparations made. A fixed amount of each DNA preparation was digested with either PstI and PvuII in separate tubes and run in an agarose gel. Nylon membranes of each gel were prepared and hybridised with radio-labelled probe which was either complementary to the plasmid or the yeast genomic integration site (Figure 8.3).

Prior to transformation, vectors were linearised at the PvuII site in the *SMR1-410* gene. This was designed to target the plasmids within the PvuII site of the *ILV2* yeast genomic sequence. Total DNA isolated and digested with PvuII, will thereby release the GUS-vector construct intact from the chromosomal DNA if site-specific integration has occurred. Figure 8.3a shows DNA of transformants and an untransformed control digested with this enzyme and probed with radio-labelled GUS sequence. The untransformed control contains weak non-specific background hybridisation bands. All transformed strains also show this banding pattern. In addition, an intensely hybridising band of approximately 10kb is visible, indicating the presence of the GUS construct and hence verifying transformation.

As previously explained, the effect of site-specific integration will provide two *ILV2* genes flanking the GUS cassette, one of which will contain the *SMR1-410* mutation conferring herbicide resistance upon the transformed cell. The restriction enzyme PstI is known to cut once within the plasmid sequence and not within the *ILV2* gene. Therefore, digestion of total DNA with PstI will split the vector, if integrated at this site, into two fragments each containing *ILV2* sequence homology. DNA of transformants and an untransformed control digested with this enzyme was probed with radio-labelled *ILV2* sequence (Figure 8.3b). The untransformed control hybridises to a single large band (>23kb) which represents the native *ILV2* gene flanked by chromosomal sequences contained within PstI restriction sites. Transformed strains also show this band, although with reduced intensity, as well as two strongly hybridising smaller bands. These 2 bands represent *ILV2* sequences separated by the digestion with PstI, indicating site-specific integration. The presence of bands corresponding to the native *ILV2* gene in transformants implies that not all *ILV2* sequences of the polyploid strain integrated a plasmid.

Figure 8.3

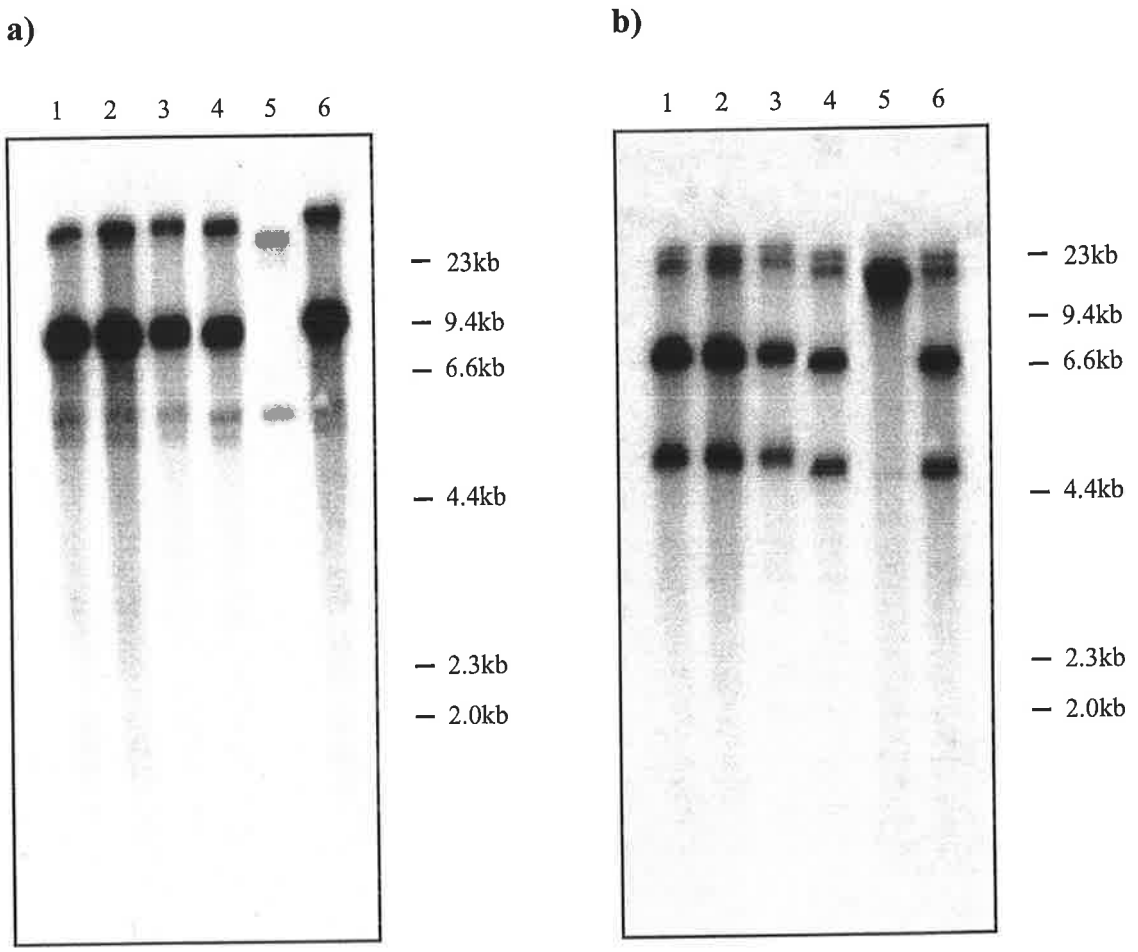


Figure 8.3 : Southern hybridisation detecting the presence and site-specific integration of GUS expression vectors in transformants of wine yeast strain 796.

Lanes 1-6 contain total yeast DNA digested with a) PvuII and b) PstI and probed with radio-labelled *GUS*and *ILV2* sequences respectively.

Lanes 1- 796 transformed with pAW222E+

2 - 796 transformed with pAW222E-

3 - 796 transformed with pAW222L+

4 - 796 transformed with pAW222L-

5 - untransformed 796 control strain

6 - 796 transformed with pAW220

Figure 8.4 : Comparison of fermentation profiles of transformed strains with an untransformed control in supplemented grape juice, as monitored by

a) *Saccharomyces* Cell Growth

b) Sugar Utilisation (Refractive Index, ^{0}Brix)

c) Ethanol Concentration (% v/v)

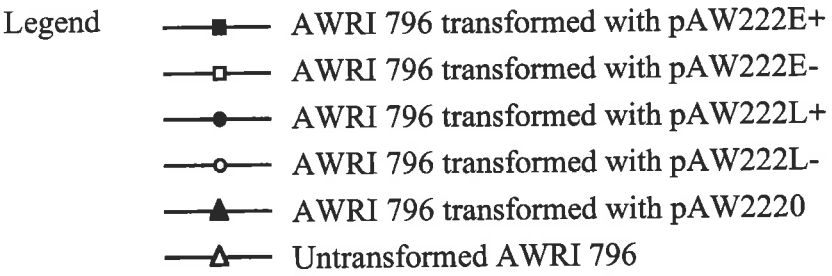
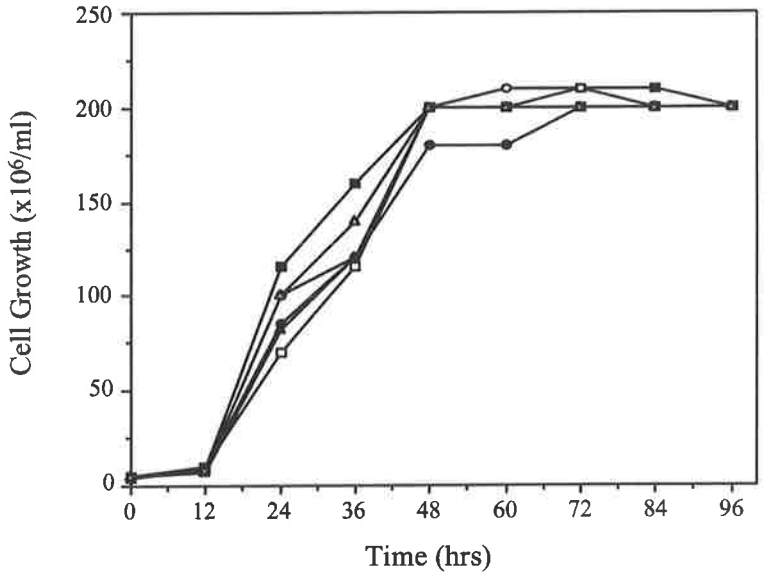
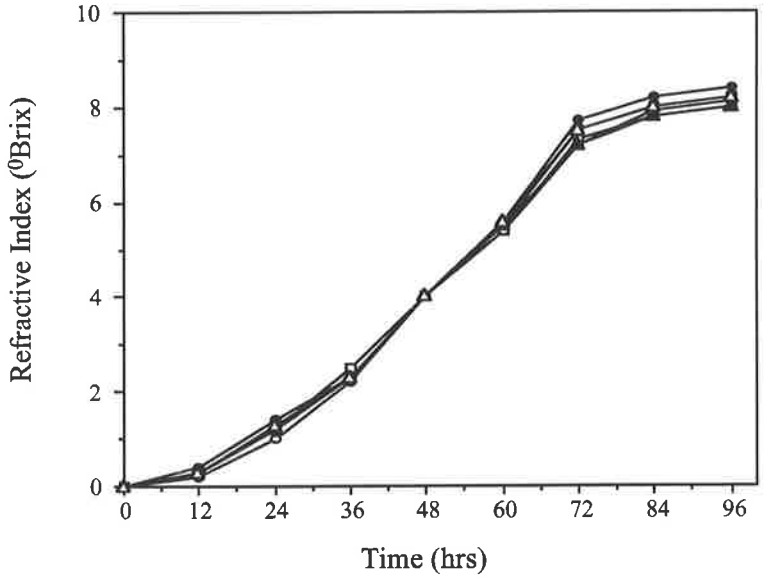


Figure 8.4

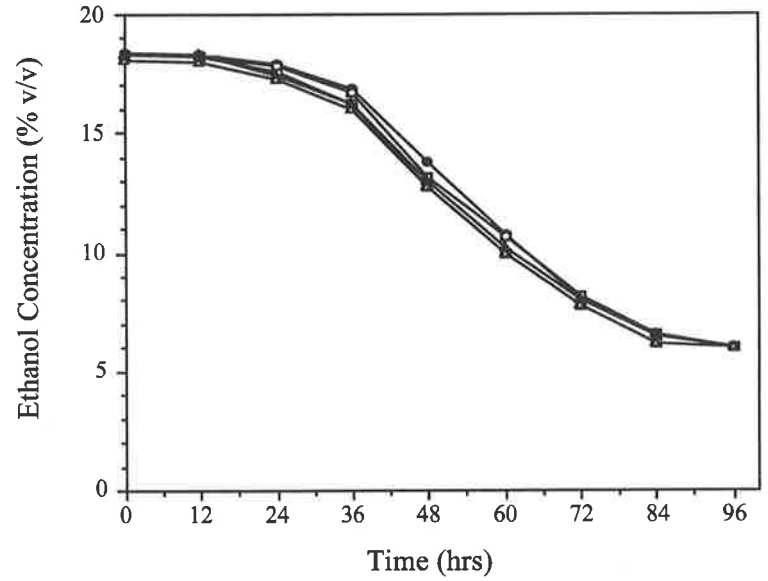
a)



b)



c)



8.4 FERMENTATION TRIALS

The transformed yeast strains were used in fermentation trials to subject the promoter sequences to the regulatory parameters under which they were selected and observe the GUS expression patterns. Besides the two novel promoter sequences, in either orientation (pAW222E+/-, pAW222L+/-), a positive control of GUS under control of the constitutive *ADH1* promoter (pAW220) and an untransformed (negative control) strain were also used. Each of the six cultures was inoculated into flasks of supplemented grape juice at a concentration of 5 x 10^6 cells/ml. Fermentations were carried out under anaerobic conditions at 18^0C with gentle agitation. Samples were taken at 12 hourly intervals and assayed for yeast growth (cell count) and the progress of fermentation was monitored by refractive index. The alcohol content was determined by near-infrared reflectance spectroscopy (Snyed *et al.*,, 1990). The relationship between the parameters for each strain was determined by comparison of these plots over time (Figures 8.4). There are no apparent differences in growth or fermentation rate between transformed and untransformed strains. Also during the time course of the fermentation cells were removed for the preparation of RNA and for use in GUS cell suspension assays.

8.4.1 GUS Cell Suspension Assays

Results of the GUS cell suspension assays are depicted in Figure 8.5. Cell culture suspensions were prepared as outlined in Chapter 3, Section 3.18.2. GUS assays were performed on cell extracts taken at 24, 48 and 72 hours during the time course of the fermentation. For all transformant types, no GUS activity was observed at the 24 hour time slot. At 48 hours some activity was observed in transformants of the *ADH1* promoter. At 72 hours GUS activity was clearly visible for the *ADH1* promoter and the two promoter sequences in sense orientation. Little or no expression was observed in the nonsense orientation promoters or the untransformed control.

8.4.2 RNA Hybridisation Analysis

Total yeast RNA was prepared from cells, electrophoresed, transferred to Hybond-N membranes and probed with radio-labelled GUS sequence as outlined in Chapter 3. The radio-labelled membranes were exposed to autoradiographic film between intensifying screens and placed at -80^0C for one week. Very little or no GUS mRNA can be observed in any lanes. Messenger RNA levels relative to the level of actin mRNA were also measured by RNA hybridisation analysis to correct for differences in total mRNA yield between samples. An example of the highest level of GUS expression observed, from the pAW220 transformant, is shown in Figure 8.6.

Figure 8.5

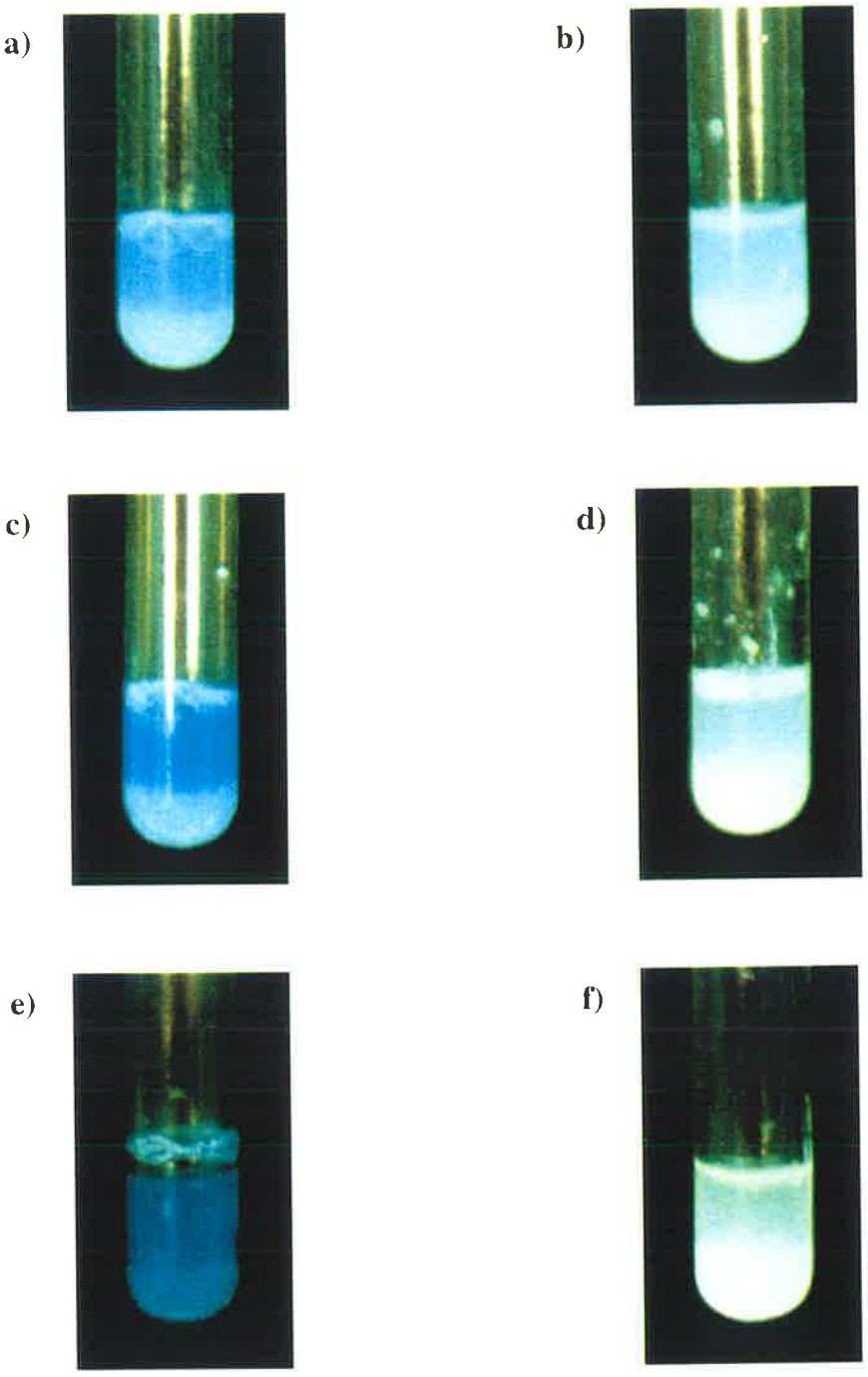


Figure 8.5 : GUS cell suspension assays of transformed wine yeast strain AWRI 796.

a) AWRI 796 transformed with pAW222E+ b) AWRI 796 transformed with pAW222E-

c) AWRI 796 transformed with pAW222L+ d) AWRI 796 transformed with pAW222L-

e) AWRI 796 transformed with pAW220 f) untransformed AWRI 796

Figure 8.6

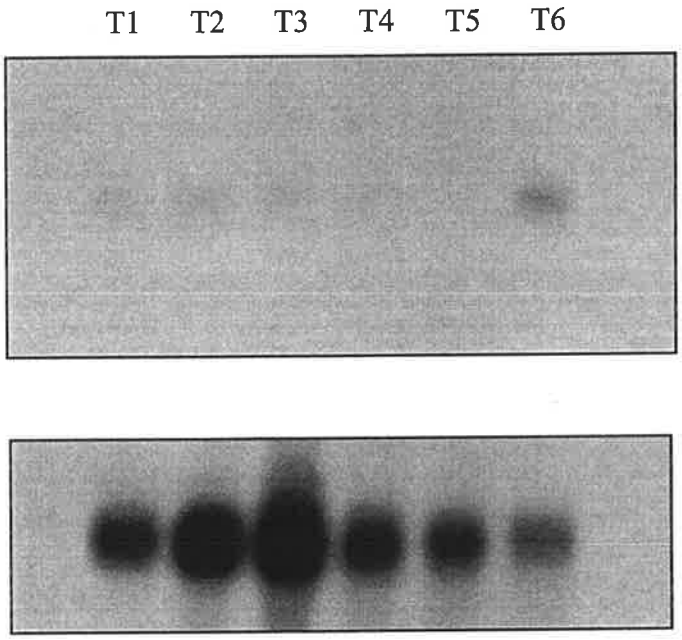


Figure 8.6 : RNA hybridisation analysis of *GUS* expression of yeast strain AWRI 796 transformed with plasmid pAW220 during an anaerobic wine fermentation.

Extraction times; T1 - 12hrs, T2 - 24hrs, T3 - 36hrs, T4 - 48hrs, T5 - 60hrs, T6 - 72hrs.

An actin control filter is also displayed.

8.5 DISCUSSION

The aim of this Chapter was to establish the effectiveness of isolated sequences to act as promoters. The plasmid pAW220 (Petering *et al.*, a/b), containing the GUS gene driven by the *ADH1* promoter, was altered to replace its promoter with a unique cloning site to produce a reporter vector capable of transforming wine yeast. Putative promoter sequences in both orientations were placed into the vector (pAW222E+/-, pAW222L+/-) and transformed into wine yeast strain. Transformants were selected on the herbicide sulfometuron methyl and GUS plate assays carried out to detect β-glucuronidase activity.

In general, the pattern of GUS activity observed, in terms of colour intensity, is as expected. The strong constitutive promoter *ADH1* produces intensely staining colonies, suggesting strong transcription and GUS activity. Putative promoter sequences in nonsense orientation produce little or no blue colour, indicating little GUS activity, although some background expression must occur in the light blue colonies. No blue colour is observed with an untransformed control strain. However, putative promoter sequences of both types, in sense orientation, appear to vary in their activity.

Possible explanations for the observed variation include: non-specific integration, in which chromosomal position has an effect; instability of the plasmid; or instability of the mRNA transcript. Non-specific integration is an unlikely explanation as randomly selected transformants all showed integration into the *ILV2* locus (Figure 8.3). Plasmid instability of the plasmid pAW220 has been shown by Petering *et al.* (1991c) within a large sample of colonies during successive subculturing of transformed strains. Also sectored colonies were detected suggesting GUS instability, by either excision of the gene by homologous recombination (Struhl *et al.*, 1979) or loss of the gene after mitotic crossing over (Roeder and Stewart, 1988).

Colonies displaying the expected GUS colour pattern were subsequently used as inocula in separate microvinification trials. Yeast growth and fermentation parameters were monitored for each strain and plotted over time. No significant differences were observed between each of the transformed strains and the untransformed control (Figure 8.4). During the time course of the fermentation, cells were removed for the preparation of RNA for hybridisation analysis and for use in GUS cell suspension assays.

A distinct positive response obtained in cell suspension assays of yeast strains containing pAW222E+, pAW222L+ and pAW220 indicates that expression of the GUS gene is achieved with these promoter constructs (Figure 8.5). Only a slight response was recorded with transformants in which the putative promoter regions are oriented incorrectly

with respect to the GUS gene (pAW222E-, pAW222L-). This confirms that fortuitous expression was not sufficient to generate a high level of GUS enzyme. Cell suspension assays were also performed on a control untransformed ferment to confirm validity of the assay. A negative response was observed with these cells (Figure 8.5).

Little β-glucuronidase mRNA was observed in the RNA hybridisation analysis (Figure 8.6). However, GUS activity was observed in the cell suspension assays, consequently some β-glucuronidase protein was present. GUS assays were performed on cell extracts taken at 24, 48 and 72 hours. For all transformant types no GUS activity was observed until 48 hours when some activity was observed in transformants of the plasmid with the *ADH1* promoter. At 72 hours GUS activity was visible for the *ADH1* promoter and the two promoter sequences in sense orientation. Either little RNA was being transcribed by strains or RNA transcripts were unstable. It is possible that GUS mRNA levels were reduced due to GUS being an *E. coli* rather than yeast reporter sequence. Petering *et al.* (1990a/c) when using the GUS gene to mark yeast cells only looked for the protein product, consequently there is no information on transcription levels of GUS in yeast. Nevertheless, a small amount of RNA was translated to a more stable protein product. Jefferson *et al.* (1986) has shown that β-glucuronidase is a very stable enzyme. However, it took 48 to 60 hours for enough protein product to accumulate and be detected enzymactically.

In conclusion, isolated sequences were shown to function as promoters since nonsense orientation controls show little or no GUS expression. However, without RNA hybridisation results, conclusive findings about regulated gene expression during fermentation were not obtained. All the sequences identified as being important in the expression of the ribosomal protein L45 (Mager and Planta, 1990; Kraakman *et al.*, 1991) were present within the 5' non-coding sequence isolated, consequently the fragment should show the expected transcriptional control. The isolated 5' non-coding sequence of the "late" gene was also able to drive the GUS reporter gene.

The experiments described thus far are only the beginning in the study of regulatory elements for wine yeast. The promoter modules isolated here require further characterisation. Identification of essential sequences necessary for *in vivo* transcriptional activity within the "late" gene promoter should be conducted by constructing deletion derivatives of this sequence. Of most interest are the UASs within both these promoter sequences which have been shown to be regulated by the changing wine fermentation parameters. These may eventually be used in the development and construction of hybrid promoters to increase the range of promoter modules available for regulated gene expression in wine yeasts.

CHAPTER 9 : GENERAL CONCLUSION AND DISCUSSION

The application of recombinant DNA technology to wine yeast strains is a relatively recent area of research but is rapidly being identified as a means of modifying the genetic properties of these strains. The potential applications of molecular genetic manipulation to the wine industry is enormous. Genetic manipulation has the capacity to replace many of the additives and processing aids of a fermentation, improve yeast fermentation performance and ultimately enhance wine flavour and aroma.

Although procedures have been developed to introduce new genetic material into wine yeasts (Petering *et al.*, 1991a/b). In many cases it will be important or indispensable to control expression of the introduced gene. This thesis describes the isolation of sequences for the control of gene expression during wine fermentations.

The results outlined demonstrate a variety of sequences that are differentially expressed at specific stages of a wine fermentation (early and late). Two sequences were subsequently isolated and analysed for use in the genetic manipulation of wine yeast. However, the elucidation of the regulatory mechanisms controlling gene expression during anaerobic wine fermentations is not a simple task.

The vector used for assaying differential expression was developed for the genetic manipulation of wine yeast strains to produce a marked strain to monitor yeasts during wine production (Petering *et al.*, 1991a/c). Problems were associated with the level of transcript produced and/or its stability and the stability of the protein product itself. Nevertheless, the sequences isolated control genes that are differentially expressed during wine fermentations and this expression is tightly linked to cell growth. These sequences offer an opportunity for controlled expression during wine fermentations.

The microvinification trials model commercial fermentations. However, variations arise in industry from winery to winery and wine to wine, depending on such factors as fermentation practice, addition of sulphur dioxide, temperature, nutrient availability, sugar concentration, available oxygen and yeast strain used. Sequences whose expression is tightly linked to cell growth means that no matter how the fermentation is conducted, controlled expression will follow the biochemical and physiological changes occurring in the cell due to the changing fermentation environment.

It may be desirable to have expression associated with cell growth and biomass accumulation while decreasing transcription as cells enter a resting stationary state. Many genes introduced to improve fermentation efficiency are required as cells are actively growing. For example, introduction and expression of a Zymocidal (killer) activity for replacement of the bioinhibitory properties of sulphur dioxide may only be required in the early stages of fermentation as yeast cells are actively dividing. As cell numbers increase above any contaminating microbial presence, killer expression could be reduced ensuring that continuous expression of the introduced gene does not burden the cell. Further, the introduction and expression of genes encoding enzymes to improve wine quality (such as wine clarity) may only be necessary early in fermentation. Alternatively, genes for flocculence and malolactic fermentations are required late within the fermentation cycle. Although expression of the "late" stage-specific clone occurs relatively early with stationary phase, an increase in transcript is observed with time and it is expected that expression continues until cell decline.

There are several situations in which it might be desirable to direct secretion of the product of a cloned gene in conjunction with its synthesis in yeast. This could be useful, for example, in the secretion of enzymes required in the must and facilitating protein purification. Therefore, an extension of the genetic manipulation of wine yeast strains requires the isolation of signal sequences for the secretion of protein products. Unlike the commonly used yeast promoters, which are often inapplicable under wine fermentation conditions, commonly used yeast signal sequences have been shown to work efficiently in wine fermentations. However, further analysis of the "late" stage-specific gene may provide a signal sequence for use in the genetic manipulation of wine yeast. A secretion signal sequence will be important in increasing the value of spent yeast as recovery and purification of proteins can be simplified. A late inducible promoter is also essential for increasing the value of spent yeast as it maximises the proportion of cells in the population which are induced to produce the heterologous product.

Ultimately the application of genetic engineering to improvement programs is dependent on the availability of suitable genes of potential importance to the industry. However, the availability of "desirable" genes is hindered by difficulties involved in isolating genes determining aromatic and flavour traits. The final challenge is to define specific targets in the winemaking process to which recombinant DNA technology can be applied. The importance of individual yeast characteristics depends on the type and style of wine to be made and the technical requirements of the winery. Strains with specific characteristics are preferred for different types of wines. For example, yeast with strong flocculent ability are

desirable in the production of sparkling wine. The choice of sequences for the alteration of a yeast strain will dictate the expression and signal sequences used. Consequently, to obtain maximum productive expression conditions each situation will need to be evaluated independently and optimised for the environment and product.

The feasibility of using genetic engineering for improving industrial strains has been demonstrated but the work has highlighted some of the short comings of our knowledge of yeast physiology, genetic manipulation and the way they interact. Ultimately, in addition to the transfer of useful genes into wine yeast strains, the genetic manipulation of wine yeasts can provide invaluable information on biological and biochemical processes involved with fermentation and provide important tools for further understanding of wine yeast biology.

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APPENDIX A

Acrylamide (30%)	:	29% acylamide
	:	1% bis-acrylamide
AE buffer	:	50mM sodium acetate pH 5.3
	:	10mM EDTA
	:	autoclave
Alkali lysing solution	:	0.5M NaOH
	:	1.5M NaCl
Ampicillin	:	50mg/ml in nanopure H_20
	:	sterile filter
Carrier DNA	:	5g salmon sperm in 100ml H_20
	:	autoclaved
Chloroform/Isoamyl alcohol	:	chloroform/isoamyl alcohol
	:	(24:1v/v)
Coomassie blue stain	:	0.5% coomassie blue
	:	50% ethanol
	:	10% acetic acid
DE52	:	75% DE52 acid washed resin
	:	25% L-broth
	:	0.1% sodium azide
Denaturing solution	:	1.5M NaCl
	:	0.5M NaOH
Denhardts III solution	:	2% polyvinyl pyrolidone 360
	:	2% ficoll 400
	:	2% bovine serum albumin
	:	sterile filter at 65^{0}C
DNA agarose gels	:	0.7-3.0% agarose in TAE buffer
	:	microwave until dissolves
DNA loading buffer	:	0.25% bromophenol blue
	:	0.25% xylene cyanol
	:	15% ficoll 4000 pH 8.0
DNA hybridisation solution	:	3ml 5xHSB
	:	2ml Denhardts III
	:	3ml 25% dextran sulphate
	:	1ml nanopure H_20
	:	0.5 ml carrier DNA
	:	0.5 ml radioactively labelled probe

DNA prehybridisation solution	:	3ml 5xHSB
	:	3ml denhardts III
	:	2ml nanopure H_20
	:	1ml 10% SDS
	:	1ml carrier DNA
Formamide/Formaldehyde	:	89µl formaldehyde (37%)
	:	250µl deionised formamide
HSB (5x)	:	3M NaCl
	:	100mM PIPES
	:	25mM EDTA
	:	adjust to pH 6.8 with NaOH
Hybridisation wash solution 1	:	2x SSC
	:	0.1% SDS
Hybridisation wash solution 2	:	1x SSC
	:	0.1% SDS
Hybridisation wash solution 3	:	0.5x SSC
	:	0.1% SDS
Hybridisation wash solution 4	:	0.2% SSC
	:	0.1% SDS
IPTG	:	0.1M isopropyl-β-thiogalactopyranoside
	:	in nanopure H_2O
	:	sterile filter
L-agar	:	1.5% agar to L-broth
	:	autoclave
L-broth	:	1% Bacto-tryptone
	:	0.5% Bacto-yeast extract
	:	1% NaCl
	:	adjusted to pH 7.2 before autoclaving
LiOAc/TE buffer	:	10mM Tris-HCl pH 7.5
	:	0.1M lithium acetate
	:	1mM EDTA
	:	autoclave
Lysis buffer	:	0.5M Tris-HCl pH 8.0
	:	20mM EDTA pH 8.0
	:	2% SDS
2M Mg^{2+}	:	1M $MgSO_47H_20$
	:	1M $MgCl_2$
	:	autoclave

MOPS/EDTA buffer (10x)	:	0.5M MOPS
	:	10mM EDTA
	:	adjust to pH 7.0 before autoclaving
Neutralising solution	:	0.5M Tris-HCl pH 7.0
	:	1.5M NaCl
	:	0.001M EDTA pH 7.2
Oligolabelling mix (2x)	:	dATP, dTTP and dGTP; 60uM each
	:	150mM Tris-HCl pH 7.6
	:	150mM NaCl
	:	30mM $MgCl_2$
	:	300µg/ml BSA
PCR reaction mix	:	1x Taq reaction mix
	:	0.06µg primer 1
	:	0.06µg primer 2
	:	0.2mM each d(ATP, CTP, GTP, TTP)
	:	1 unit Taq polymerase
	:	water to final volume
20% PEG/2MNaCl	:	20% polyethylene glycol 6000
	:	2M NaCl in SM buffer
PEG reagent	:	40% polyethylene glycol 4000
	:	0.1M lithium acetate
	:	10mM Tris-HCl pH 7.5
	:	1mM EDTA
	:	autoclave
Phage buffer	:	20mM Tris-HCl pH 7.4
	:	100mM NaCl
	:	10mM $MgSO_4$
	:	autoclave
Phenol	:	Phenol equilibrated in 1 x TE and
	:	0.1% hydroxyquinoline
Phenol/Chloroform/Isoamylalcohol	:	phenol:chloroform:isoamylalcohol
	:	(25: 24:1 v/v)
Plasmid preparation solution 1	:	50mM glucose
	:	25mM Tris-HCl pH 8.0
	:	10mM EDTA
	:	autoclave
Plasmid preparation solution 2 (freshly prepared)	:	0.2N NaOH
	:	1% SDS
Polyacrylamide electrode buffer (5x)	:	15g/l Tris base
	:	72g/l glycerine
	:	5g/l SDS

Polyacrylamide separating gel	:	1.3ml acrylamide (30%)
	:	100µl 10% SDS
	:	6.1ml distilled H_2O
	:	50µl 10% APS (freshly made)
	:	2.5ml 0.5M Tris-HCl pH 6.8
	:	10µl 10% TEMED
Polyacrylamide stacking gel	:	4.0ml acrylamide (30%)
	:	100µl 10% SDS
	:	2.5ml 1.5M Tris-HCl pH 8.8
	:	50µl 10% APS (freshly made)
	:	3.35ml distilled H_2O
	:	5µl 10% TEMED
PPO (20%)	:	20% 2,5 diphenyloxazole
	:	in galacial acetic acid
R40	:	40µg/ml RNase in TE buffer
Radioactive first strand reaction mix	:	4µl 5 x reaction buffer
	:	1µl 0.1M DTT
	:	1µl 10mM mixed dNTP stock (-dCTP)
	:	2µl (α^{32}P) dCTP
RNA buffer A	:	294ml 10x MOPS/EDTA
	:	706ml nanopure H_2O
	:	autoclave
RNA denaturing gel	:	0.7g agarose
	:	36ml nanopure H_20
	:	5ml MOPS/EDTA buffer
	:	microwave to melt before adding
	:	9ml formaldehyde (37%)
RNA hybridisation solution	:	2ml denhardts
	:	1ml 10% SDS
	:	1ml carrier DNA
	:	5ml 20x SSPE
	:	10ml formamide
RNA loading buffer	:	322ml RNA buffer A
	:	178ml formaldehyde (37%)
	:	500ml deionised formamide
	:	3mg xylene cyanol
	:	3mg bromocresol green
	:	250mg sucrose
		sterile filter
Sephadex G-100	:	100g sephadex to 300ml TE
	:	shaking 2hrs at 65^{0}C

SD medium	:	0.67% Bacto-yeast N base without aa
	:	2% glucose
	:	1.5% agar
	:	autoclave
SDS (10%)	:	100g sodium dodecyl sulphate
	:	900ml nanopure H_2O
SM buffer	:	0.1% gelatin in phage buffer
	:	autoclave
SOB medium	:	4% Bacto-tryptone
	:	1% Bacto-yeast extract
	:	10mM NaCl
	:	2.5mM KCl
	:	autoclave
SOC	:	1ml SOB
	:	10µl 2M Mg^{2+}
	:	7µl 50% glucose
SSC (20x)	:	3.0M NaCl
	:	0.3M sodium citrate
SSPE (20x)	:	3.6M NaCl
	:	0.2M NaH_2PO_4
	:	0.02M EDTA
	:	autoclave
STE buffer	:	150mM NaCl in TE
	:	autoclave
Stripping solution	:	0.1% SDS
	:	2mM EDTA pH 8.0
Sulfometuron methyl	:	1mg/ml sulfometuron methyl
	:	dissolved in acetone
TAE buffer	:	40mM Tris-acetate pH 7.5
	:	1mM EDTA
TE buffer	:	10mM Tris-HCl pH 7.5
	:	1mM EDTA
	:	autoclave
TFB buffer	:	10mM MES
	:	45mM $MnCl_2$
	:	10mM $CaCl_2$
	:	3mM $HACoCl_3$
	:	sterile filter

TB top agarose	:	0.5% Bacto-yeast extract
	:	1% Bacto-tryptone
	:	0.5% NaCl
	:	0.7% agarose
	:	adjust to pH 7.0 before autoclaving
X-gal	:	2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in dimethyl formamide
	:	sterile filter
X-GLUC	:	10mg/ml 5-bromo-4-chloro-3-indolyl glucuronide in dimethyl formamide
	:	sterile filter
YPD medium	:	1% Bacto-yeast extract
	:	2% Bacto-peptone
	:	2% glucose
Zymolase buffer	:	1.2M sorbitol
	:	0.1M sodium citrate
	:	0.06M EDTA
	:	adjust to pH 7.0 before autoclaving

APPENDIX B

PCR Program U55	step 1	4 minutes, 94^{0}C
	step 2	1 minute, 94^{0}C
	step 3	2 minutes, 55^{0}C
	step 4	2 minutes, 72^{0}C
	step 5	repeat steps 2 - 4, 35 times
	step 6	10 minutes, 72^{0}C
	step 7	5 minutes, 25^{0}C
	step 8	hold 25^{0}C
PCR Program U50	step 1	4 minutes, 94^{0}C
	step 2	1 minute, 94^{0}C
	step 3	2 minutes, 50^{0}C
	step 4	2 minutes, 72^{0}C
	step 5	repeat steps 2 - 4, 35 times
	step 6	10 minutes, 72^{0}C
	step 7	5 minutes, 25^{0}C
	step 8	hold 25^{0}C
PCR Program U45	step 1	4 minutes, 94^{0}C
	step 2	1 minute, 94^{0}C
	step 3	2 minutes, 45^{0}C
	step 4	2 minutes, 72^{0}C
	step 5	repeat steps 2 - 4, 35 times
	step 6	10 minutes, 72^{0}C
	step 7	5 minutes, 25^{0}C
	step 8	hold 25^{0}C
PCR Program SEQ55	step 1	5 minutes, 94^{0}C
	step 2	30 seconds, 94^{0}C
	step 3	30 seconds, 55^{0}C
	step 4	1 minute, 72^{0}C
	step 5	repeat steps 2 - 4, 14 times
	step 6	30 seconds, 94^{0}C
	step 7	1 minute, 72^{0}C
	step 8	repeat steps 6 - 7, 14 times
	step 9	30 minutes at 4^{0}C
	step 10	hold 25^{0}C