Analysis of a set of stationary phase genes in *Saccharomyces cerevisiae*

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by

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Dedicated to my Mum, Sheila Hather,
from her son, Richard.

To Rob, my brother who was there, when times were hard

The Truth is out-there,
Fox Mulder
X-files

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Abstract

This thesis describes the characterisation of a set of six genes of the baker's yeast *Saccharomyces cerevisiae*, that are induced when a molasses-grown culture reaches the end of rapid fermentative growth. The rationale for the isolation of such genes was that their promoters may be useful in a biotechnological application, where the regulated expression of heterologous proteins is required. Additionally, their analysis should provide information about how yeast cells adapt to changing environmental situations. Therefore the aims of this project were to identify the unknown members of this set of genes and to determine the transcriptional regulatory mechanisms acting on all six.

Six cDNA clones, which showed differential hybridisation to mRNAs of exponential and stationary phase cells, were used as probes to recover the cognate genes from a library of yeast genomic DNA fragments carried in the bacteriophage vector, λEMBL3. Two had been identified as the previously described genes, *HSP26* and *HXK1* encoding the 26kDa heat shock protein and the glucose-repressible hexokinase, respectively. A further two, previously undescribed genes, encode a second small, 12kDa, stress-induced protein (*HSP12*) and a thiamine biosynthetic enzyme (*THI4*). DNA sequence analysis carried out in this study has characterised the two remaining genes as *HXT4=LGT1*, a low affinity hexose transporter and a new thiamine regulated gene, *THI5*.

Transcription of these six genes has been examined under a variety of growth conditions and in various mutant backgrounds. The two heat-shock genes, *HSP12* and *HSP26*, are subject to glucose control via a cAMP-dependent protein kinase and become induced when the cells experience stress or starvation; presumably they have a role in maintaining the integrity of the cell under these conditions. The genes involved in hexose metabolism, *HXK1* and *HXT4*, are regulated by glucose repression through the *TUP1/CYC8/SNF1* pathway and are derepressed by exhaustion of glucose from the growth medium; their products enable the cell to utilise alternative hexoses as growth substrates. Both of the thiamine genes, *THI4* and *THI5*, are subject to repression by exogenous thiamine and become derepressed on depletion of the vitamin from the growth medium. Thus, although these genes show apparent coordinate induction they are regulated via different transcriptional control mechanisms.

DNA sequence analysis revealed the *THI5* gene to encode a second thiamine biosynthetic enzyme homologous to the product of the *Schizosaccharomyces pombe* gene *nmt1*. *THI5* is present within the yeast genome in multiple copies located on

chromosomes VI (*THI5*), X (*THI11*) and XIV (*THI12*). Evidence is presented to show that all three copies potentially express a functional product. Likely mechanisms by which the multiple copies of this gene have arisen and subsequent conclusions about the evolution of the yeast genome are discussed.

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Abbreviations and symbols

A	Adenine
Ac	acetate
Ala	alanine
Amp	ampicillin
cAMP	cyclic adenosine triphosphate
Arg	arginine
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CIAP	alkaline phosphatase, calf intestine
Cys	cysteine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EtOH	ethanol
G	guanine
Gln	glutamine
Glu	glutamate
His	hisitidine
HCl	hydrochloric acid
Ile	isoleucine
IPTG	ß-D-isopropyl-thiogalactopyranoside
KCl	potassium chloride
Kb	kilobase
kDa	kilodalton
λ	lambda
l	litre
Leu	leucine
LiAc	lithium acetate
Lys	lysine
M	molar
Met	methionine
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messanger RNA
MW	molecular weight
nt	nucleotide
NaCl	sodium chloride
NaOAc	sodium acetate
NADH	nicotinamide adenine dinucleotide (reduced form)

p	protein/plasmid
P	phosphorus
Phe	Phenylalanine
RE	restriction endonuclease
RNA	ribonucleic acid
RNase	ribonuclease
S	sulphur
SDS	sodium dodecyl sulphate
T	thymine
TE	Tris-EDTA buffer
Trp	tryptophan
U	uracil
µg	microgram
µl	microlitre
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-ß-galactopyranoside

Chapter One:
Introduction

1.1 *Saccharomyces cerevisiae* as a model eukaryote

S. cerevisiae is a unicellular, eukaryotic micro-organism, which responds to alteration in its external environment, by changes in metabolism and gene expression. The majority of these responses are controlled at the level of mRNA production, which makes *S. cerevisiae* a useful model organism in which to examine transcriptional regulatory mechanisms. Under optimal nutritional conditions cell culture mass doubles every ninety minutes, as each cell forms a bud-appendage into which a full complement of daughter chromosomes moves during mitotic division. The enlarged bud is pinched off yielding a single daughter cell, which increases to the size of the parent and then buds itself. Yeast exhibits a typical eukaryotic cell cycle and can exist in either a haploid or diploid state, with haploid cells as either of two mating types, a or α.

Yeast cells contain typical eukaryotic membrane bound cell organelles i.e. endoplasmic reticulum, golgi apparatus, mitochondria, nucleus, secretory vesicles and vacuole contained within a phospholipid bilayer plasma membrane. This cell membrane is linked to an outer cell wall composed of ß-glucans (a polymer of D-glycopyranose groups, linked a and ß(1-3)) and chitin (a polymer of *N*-acetylglucosylamine, linked ß(1-4)), with an outer surface that contains highly immunogenic mannoproteins. Between this outer mannoprotein layer and the plasma membrane is the more open periplasmic region, which contains a number of degradative enzymes i.e. sucrose hydrolyzing invertase and the phosphate-releasing enzyme, acid phosphatase.

The *S. cerevisiae* haploid genome, comprises 1.4x 10^7 DNA base pairs, distributed among sixteen discrete chromosomes, within a membrane bound nucleus. The yeast genome project aims to sequence the entire genome within the year 1995 and complete DNA sequences of chromosomes II, III, VI, IX and XI are already available (June 1995- EMBL database) (Feldmann, 1994; Oliver *et al.*, 1992). The majority of yeast genes are transcribed into a monocistronic mRNA product, where control elements 5' and 3' to this coding region effectively define the gene boundary. These control elements comprise specific base sequences which can be several hundred bases 5' of the transcription start site and act by binding specific cellular

proteins that regulate transcription (Guarente, 1984). Binding of positive control proteins to upstream activating sequences (UAS) increases the transcription rate, whereas binding of negative control proteins to a upstream repressing sequence (URS) decreases the rate of transcription. The interaction of these elements results in a complex pattern of regulation, tuned to respond to the changes in the external environment. Within the promoter region, there are two other essential components; TATA sequences which are required for the accurate positioning of the transcriptional complex for the start of transcription and an initiator element. TATA sequences are usually located between twenty to forty base pairs 5' to the transcription start site, whereas the remaining initiator element encompasses the transcription start site and determines precisely where transcription begins.

The rate at which a eukaryotic gene is translated is dependent upon a number of factors, in particular the abundance of the respective mRNA in the cytoplasm, where the level of an mRNA transcript is dependent not only upon its rate of synthesis but also on its degradation (an essential prerequisite for the flexibility of gene expression). Eukaryotic mRNA species have a specific structure, with untranslated sequences preceding and following the protein coding region. The 5' terminus is modified by the addition of a methyl-guanine residue, referred to as the 5' cap, which is thought to aid ribosome attachment to the mRNA chain so that translation commences at the first AUG codon and is required for efficient translation. It also appears to aid in the stability of the mRNA molecule by protecting against 5' exoribonuclease activity (Piper *et al.*, 1987). At the 3' end of the mRNA molecule is a long stretch of between 50 to 200 adenine residues, termed the poly A tail, which is not specified by genomic DNA. The poly A tract is not essential for mRNA synthesis or export from the nucleus, however, it does appear to confer protection against 3' exonuclease attack, where the degree of resistance to degradation appears to be dependent upon the length of this poly A tail (Littauer and Soreq, 1982). Several factors affect mRNA stability, with the rate of decay for some mRNA species altered by physiological signals or changes in growth rate (Brawerman, 1987; Brown, 1989).

1.2 Growth of *S. cerevisiae* in batch culture

The particular pattern of growth observed for a given yeast culture is critically dependent upon the nature of the growth substrate and aeration conditions. Where under defined culture conditions this pattern of growth is characteristic for a specific yeast species, with distinct phases (Fiechter, Kappeli and Meussdoerffer, 1987; Kappeli, 1986; Lievense and Lim, 1982).

Under conditions of aerobic growth on glucose, *S. cerevisiae* displays six distinct phases of growth; lag, respiro-fermentative, diauxic lag, respiratory, stationary phase and death (figure 1.1) (Lewis *et al.*, 1993). The phases of growth observed are characterized by distinct physiological events, where in the initial lag period adaptive changes occur, upon the addition of the inoculum to fresh medium. In the second phase, biomass increases exponentially associated with glucose depletion and ethanol accumulation, where high external glucose concentrations cause inhibition of respiratory functions (Crabtree effect) so that cells grow by anaerobic fermentation, with ethanol and carbon dioxide produced as byproducts (Barford and Hall, 1979; DeDeken, 1966). On depletion of glucose the inhibition of respiration is released and a secondary, slower growth phase (non-fermentative) is observed where accumulated ethanol is utilised as the substrate. The diauxic lag between these two growth phases reflects the period of time required for the release of genes from glucose repression and adaptation from fermentative to respiratory metabolism (Haarasilta and Oura, 1975). On depletion of all available carbon sources, the cells enter a distinct stationary phase where growth ceases (Drebot *et al.*, 1990; Werner-Washburne *et al.*, 1993).

In contrast, cultures grown on glucose under anaerobic conditions show no diauxic shift and grow only by fermentative metabolism, accumulating unusable ethanol as a byproduct (i.e. brewing). In aerobic cultures growing on other carbon sources which are also metabolised via the glycolytic pathway, but which do not exert the catabolite or respiration repressing activity of glucose (e.g. galactose and raffinose), the active growth period involves a mixed respiro-fermentative metabolism, where respiratory functions, sugar uptake and metabolism are all fully active. Under these conditions, the transition into purely respiratory growth does not require a period of depletion and hence the diauxic growth pattern is not observed. In cultures that only contain carbon sources that cannot be metabolised by fermentation (e.g. glycerol, ethanol and lactate), growth is purely respiratory and can therefore only take place under aerobic conditions. These cultures, exhibit only a single phase of growth and then enter into stationary phase upon exhaustion of the substrate. The studies in this thesis are concerned with genes that change in their levels of expression as cultures transition from one growth stage to another, particularly during the diauxic lag and on entering into stationary phase.

Differences in the pattern of protein expression between yeast cells in the first period of exponential growth and those in stationary phase are due to responses to the change in the external environment (Boucherie, 1985; Iida and Yahara, 1984). *S. cerevisiae* cells enter stationary phase when starved of carbon source or other nutrients including nitrogen, phosphorus and sulphur (Lillie and Pringle, 1980).

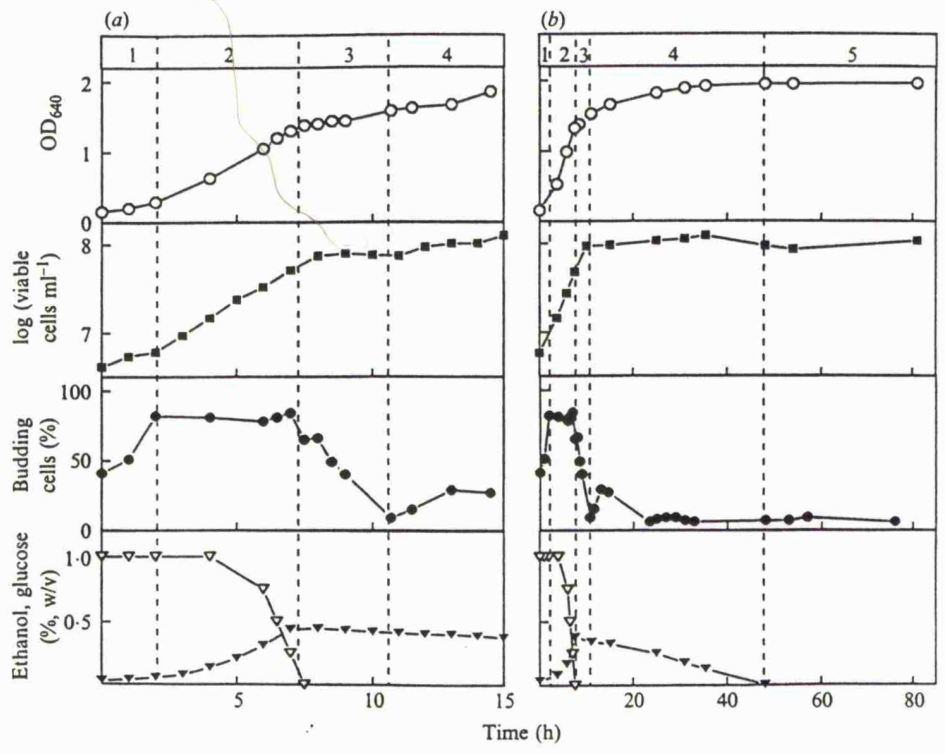


Figure 1.1: Growth of *S. cerevisiae* A9 in batch culture: (a) 0-15 hours, (b) 0-75 hours. O, $O.D_{640}$; ■, log viable cells ml^{-1}; ●, percentage of budding cells; ▼, [ethanol]; ▽, [glucose] (after Lewis *et al.*, 1993). Data is from a representative experiment. Growth phases are indicated by vertical dashed lines; (1), initial lag phase; (2), respiro-fermentative phase; (3), diauxic lag phase; (4), respiratory phase; (5), stationary phase, and (6) death phase.

However, these cells retain their ability to resume growth dependent upon the availability of the appropriate nutrients. Stationary phase cells are invariable found as single, unbudded cells containing unreplicated nuclear DNA. This unbudded, prereplicative state is characteristic of a cell that has yet to perform the start step in G_1 of the mitotic cycle. However, stationary phase cells are distinctly different from those in a G_1 arrested state, in that 'stationary' cells exhibit a regulated arrest of cell proliferation, increased thermotolerance, elevated levels of storage carbohydrates and the induction of certain genes (Drebot, *et al.*, 1990).

During the transition from exponential to stationary phase, major changes in cell metabolism and physiology occur as the cell prepares for starvation. Proteases accumulate in various subcellular locations and polyphosphate concentration increases in the vacuole. Total phospholipid synthesis decreases, with an accompaniment change in the relative composition of the plasma membranes and cell wall thickness. Storage carbohydrates, trehalose (a non-reducing disaccharide of two D-glucose molecules) and glycogen (a polymer of glucose, linked by predominately α-1,4-glycosidic bonds, with α-1,6-glycosidic bonds approximately every ten residues) also accumulate within the cytoplasm, with glycogen concentration peaking upon entry into the diauxic lag, whereas trehalose accumulation begins at the diauxic lag and continues until cells enter the stationary phase, where levels then decline (Lillie, and Pringle, 1980). The intracellular concentrations of accumulated glycogen or trehalose are dependent upon the growth status of the cell and also vary in response to stresses including heat shock, limitation of nitrogen, sulphur, phosphorus or carbon sources (Francois, Neves and Hers, 1991; Panek and Panek, 1990; Slaughter and Normura, 1992; Torres, Eymard and Panek, 1991; Wiemken, 1990). It has been suggested that trehalose protects cells from dessication or other stresses and that glycogen serves as a reserve carbohydrate which is mobilized to supply glucose during times of nutritional stress (Panek and Panek, 1990; Wiemken, 1990). Although recent evidence has shown that there is a direct correlation between trehalose concentration and thermotolerance in stationary phase cells, long term viability is not always correlated with intracellular accumulation of trehalose (Slaughter and Normura, 1992).

Stationary phase cells exhibit reduced transcription and translation rates, where most individual mRNA species abundant in exponential cells are absent after the diauxic lag and in stationary phase cells (Werner-Washburne *et al.*, 1989). However, not all transcripts decrease in abundance as cells enter into stationary phase, with some genes induced upon entry into stationary phase. These include *UBI4*, a member of the ubiquitin gene family, members of which are involved in the selective

degradation of intracellular proteins (Finley, Ozkaynak and Varshavsky, 1987; Tanaka, Matsumoto and Toh-e, 1988), *BCY1* (Toda *et al.*, 1987) (see section 1.3), *CTT1* (encoding catalase T1) (Bissinger *et al.*, 1989), heat shock genes *SSA3*, *HSP12* and *HSP26* (see section 1.4) and other as yet unidentified genes. Induction of *UBI4*, results in a several fold accumulation of the mRNA after the diauxic lag, which then decreases as the yeast cells enter stationary phase (Tanaka *et al.*, 1988). The *UBI4* gene is strongly induced by starvation, high temperatures and other stresses (Ozkaynak *et al.*, 1987) and *ubi4* strains are sensitive to both carbon and nitrogen limitation (Finley *et al.*, 1987). Since ubiquitin is necessary for the degradation of abnormal proteins, there is probably an increased demand for ubiquitin during periods of stress i.e. after heat shock. As ubiquitin is fused with proteins incorporated into the ribosome's, it is possible that ubiquitin acts also as a molecular chaperone (Finley, Bartel and Varshavsky, 1989). As the shift to stationary phase is associated with major alterations in cell metabolism and physiology, the principle mechanisms involved will be reviewed below.

1.2.1 Carbon metabolism: An overview

The metabolism of *S. cerevisiae* has been extensively examined, however, a complete description of the interactions involved within the various metabolic pathways is far from complete. Presented below is an overview of the current understanding of the main metabolic pathways found within *S. cerevisiae*. All cellular mechanisms that perform work i.e. biosynthetic, osmotic and mechanical use ATP as energetic currency, provided by the oxidation of organic molecules which also act as carbon sources for biosynthesis. The coupling of the energy and carbon metabolism pathways therefore requires elaborate control mechanisms as yet not fully defined (see section 1.3).

S. cerevisiae is able to utilise a variety of carbon sources including alcohols, hexoses, pentoses and some oligosaccharides (Polakis and Bartley, 1965; Polakis, Bartley and Meek, 1965; Stewart and Russell, 1983) as well as the respiratory substrates ethanol, acetic acid, pyruvate, lactate and glycerol. However, the preferred substrate is glucose (Barnett, 1976; Barnett, 1981; Wills, 1990). In general disaccharides and some oligosaccharides are not transported into the yeast cell but are hydrolysed by extracellular enzymes into their constituent monosaccharides, which are then assimilated (Santos *et al.*, 1982). Although sucrose is generally thought to be hydrolysed in the periplasmic space by the extracellular enzyme invertase (a glycosidase), it has, however, also been reported to be assimilated directly into the cell (Barford *et al.*, 1993).

The primary carbon source, glucose is an important molecular signal for yeast cells, influencing cell cycle progress via the RAS/cAPK pathway (see section 1.3), regulating gene expression at the level of transcription and activating the plasma membrane bound proton-pumping ATPase, which results in the uptake of calcium and potassium ions (Suomalainen and Oura, 1971). Utilization of intracellular hexoses (e.g. glucose or fructose), derived either from the free external sugar (transported into the cell) or from oligosaccharides by the action of the appropriate glycosidase is via the glycolytic pathway leading to the TCA cycle. Intracellular hexoses are phosphorylated by one of three different kinases; hexokinase A or P-I (encoded by *HXK1*) which phosphorylates glucose and fructose, hexokinase B or P-II (encoded by *HXK2*) which exhibits similar rates of phosphorylation on the two sugars and glucose kinase which acts only on glucose (Entian and Frohlich, 1984; Entian *et al.*, 1984). Hexokinase P-II (*HXK2*) has been shown to be a major component in both glucose transport and catabolite repression (see section 1.3) (Frohlich, Entian and Mecke, 1985). Hexokinase P-I (*HXK1*), is associated with late exponential growing cells and has been reported to be able to substitute for hexokinase P-II in glucose repression if it is expressed under the same conditions of high sugar concentration (Rose, Albig and Entian, 1991).

Glucose-6-phosphate forms the first part of a pool of hexose monophosphates, which can be interconverted by the action of appropriate isomerases allowing the entry into the glycolytic pathway. Fructose enters into the glycolytic pathway via the conversion of fructose-6-phosphate into fructose-1,6-bisphosphate catalysed by phosphofructokinase (a key regulatory enzyme in glycolysis). Pyruvate produced in the common glycolytic pathway may follow either of two principal routes, depending upon environmental conditions. Under aerobic conditions, pyruvate is oxidised by the TCA cycle, whereas during anaerobiosis it is decarboxylated to acetaldehyde and reduced to ethanol by alcohol dehydrogenase I (encoded by *ADH1*) (Ciricacy, 1975; Denis, Ciriacy and Young, 1981). When acetaldehyde metabolism is blocked or diverted into the production of intermediates for other biosynthetic pathways, the minor fermentation product glycerol is produced by the reduction of dihydroxyacetone phosphate to glycerol phosphate and then to glycerol (Gancedo, Gancedo and Sols, 1968). The initial reduction reaction to glycerol phosphate, results in the regeneration of NAD+, by the oxidation of NADH.

The end products of fermentation, ethanol or glycerol can only be utilized in the presence of oxygen. Growth under these conditions is dependent upon the oxidation of intermediates via the tricarboxylic acid (TCA) cycle (leading to pathways where catabolic and anabolic intermediates are interconverted) (Fraenkel, 1982; Wills,

1990). Accumulated ethanol and other non-fermentable carbon sources (e.g. glycerol and lactate) enter the yeast cell by rapid passive diffusion. Ethanol is then utilized via a series of intermediates and enters into the TCA cycle via acetyl CoA, after the de-repression of the glucose repressible alcohol dehydrogenase II (*ADH2*). The alternative carbon source, glycerol is utilised by phosphorylation/oxidation only under aerobic conditions (Barnett, 1976; Gancedo *et al.*, 1968), where the main control appears to be glucose repression of respiratory enzymes (Sprague and Cronan, 1977). Yeast cells can also utilize L-lactate as a carbon source, after transport into the cell either by passive diffusion or more likely by an active transport mechanism coupled to a proton gradient, which has been reported to be inducible and subject to glucose repression control (Leao and van Uden, 1986). Lactate is oxidised to pyruvate by the inducible L-Lactate:cytochrome *c* oxidoreductase. Utilisation of these alternate carbon sources requires the reversal of the glycolytic pathway for the synthesis of hexose monophosphates, termed gluconeogenesis. Gluconeogenic enzymes are repressed and inactivated by glucose and other sugars (Haarasilta and Oura, 1975).

Yeast cells also require a number of other essential nutrients i.e. nitrogen, sulphur and potassium (Berry and Brown, 1987). Active growth requires the uptake of nitrogen, either in the form of ammonium ions or amino acids, for the synthesis of protein, nucleic acids and other nitrogenous components of the cell. In fact the eight amino acids arginine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, serine and threonine are actively assimilated via a regulated transport uptake. However, the uptake of proline by proline permease is repressed by other amino acids and ammonium ions (Horak, 1986; Island, Naider and Becker, 1987). Although yeast cells are able to synthesize most vitamins *de novo* they actively assimilate these components from their environment, by transport systems specific for the individual vitamins e.g. thiamine (see section 1.5), riboflavin, vitamin B_6 and biotin (Iwashima, Wakabayashi and Nose, 1977).

1.2.2 Carbon transport mechanisms

Transport of essential nutrients across the plasma membrane must occur if the yeast cell is to supply the various metabolic pathways which are necessary for cell maintenance and growth. These processes are mediated via a series of specific transport pathways, which are regulated via distinct mechanisms (Cartwright *et al.*, 1989). Within *S. cerevisiae* there are three basic types of solute transport processes, the first is free diffusion, where a solute passes into the cell without the involvement of proteins within the plasma membrane. The two remaining processes involve

membrane bound proteins, where the movement of soluble molecules across the membrane, takes place down a concentration gradient (facilitated or non-concentrative diffusion) or against a concentration gradient, a process that requires metabolic energy and hence is termed 'active' transport.

In the natural environment, *S. cerevisiae* is almost entirely found on any sugary substratum e.g. grapes, which have a varying total sugar content (upto 40% (w/v)), hence sugar transport must function over a large range of concentration and osmolarities. Therefore, there exists a complex sugar transport system, with numerous elements and levels of control. Cytoplasmic glucose concentrations are normally low as the rate of transport is tightly regulated, where the amount of glucose taken into the cell is equivalent to that which consumed, hence avoiding ATP depletion by sugar kinase activity. In *S. cerevisiae* two affinities for glucose uptake have been identified by kinetic analysis; 'low' affinity uptake, active in high external glucose concentrations and 'high' affinity uptake, active in low external glucose concentrations. Transport of sugars into the yeast cell is the primary step in sugar metabolism, and is the common step in the modulation of and response to glycolytic flux and cellular activities of the cell. The rate of glucose utilization is governed by the number and distribution of hexose transporters within the plasma membrane. These transporters appear to be involved in the process of sensing external glucose concentration either alone or in combination with the first enzymatic step of glycolysis, sugar phosphorylation. The loss of any of the irreversible steps of glycolysis, i.e. hexokinase, phosphofructokinase or pyruvate kinase, prevents expression of any transporter activity. Of the three irreversible steps in glycolysis, the removal of phosphofructokinase has the most dramatic effect, with loss of both 'low-' and 'high-' affinity uptake (Bisson and Fraenkel, 1983). Sugar transport is associated with phosphorylation activity, by hexokinase P-I, P-II and glucokinase. Hexokinase P-II (encoded by *HXK2*) appears to be constitutively expressed and although there is no detectable change in growth on glucose or fructose in the *hxk2* mutant (Gancedo, Clifton and Fraenkel, 1977; Muratsubaki and Katsume, 1979), a loss of glucose repression is observed (Ma and Botstein, 1986). Hexokinase P-II exhibits a protein kinase activity, capable of both autophosphorylation and phosphorylation of other proteins, whose kinase activity has been shown to influence the kinetics of 'low' affinity glucose or fructose transport (Bisson and Fraenkel, 1983).

Numerous putative transporters have been identified by their ability to complement or suppress the *snf3* phenotype, which was found to be deficient in 'high' affinity glucose and fructose transport (Bisson *et al.*, 1987). To date nine putative transport proteins have been identified in *S. cerevisiae* indicating that there is a large

degree of redundancy within the transport system. The following set of glucose transporters encoded by the genes *SNF3* and *HXT1*, *HXT2* and *HXT4* will be discussed below.

The *SNF3* gene encodes a protein of 884 amino acid residues, localised to the plasma membrane, which confers a 'high' affinity glucose uptake (Bisson *et al.*, 1987). From the primary amino acid sequence, predicted membrane spanning domains typical of a transport protein are detected, however, the carboxyl-terminus (C-terminus) is unusually long (approximately 303 amino acid residues), with at least 150 residues of this tail required for *SNF3* function. The C-terminus also shows several other distinctive features such as numerous sites for phosphorylation by a cAMP dependent protein kinase and a repeated sequence, postulated to be involved in activation or stability of carrier activity (Bisson *et al.*, 1993).

HXT1, *HXT2* and *HXT4* are members of a multi-gene family and are each capable of complementing the *snf3* phenotype. Transporters encoded by this family show differing times and levels of expression with varying substrate specificities (Ozcan and Johnston, 1995). Multicopy expression of *HXT1* restores 'high' affinity glucose uptake in the *snf3* mutant. The *HXT1* encoded protein appears to play a specialised role within the cell that is as yet undefined. Expression of *HXT1* is reduced on entry into stationary phase, correlated with glucose depletion (Lewis and Bisson, 1991). *HXT2* encodes a protein 541 amino acid residues in length, which restores 'high' affinity glucose uptake in the *snf3* mutant, when expressed at high copy number and is reported to be expressed at high levels upon glucose derepression. In the double mutant, *hxt2 snf3* glucose transport is severely defective, but fructose or mannose uptake is unaffected, which indicates that *HXT2* is involved only in glucose uptake (Kruckeberg and Bisson, 1990). Expression of both *SNF3* and *HXT2* is repressed by high extracellular concentrations of glucose, which suggests that these 'high' affinity transporters are responsible for scavenging residual glucose from the external cell environment.

HXT4 (also called *LGT1*) encodes a 'low' affinity glucose transporter which complements the *rag*$^-$ phenotype in *Kluyveromyces lactis* (Prior *et al.*, 1993). The *rag*$^+$ phenotype was initially characterized in *K. lactis*, as cell growth on glucose in the presence of mitochondrial inhibitors (Goffrini *et al.*, 1989). *HXT4* encodes a protein of 576 amino acid residues, which is unique as it is the only transporter identified that affects both glucose and galactose uptake. Over-expression of *HXT4* results in an increase in both 'high' and 'low' affinity uptake of glucose (Ko, Liang and Gaber, 1993).

Glucose transport is regulated via a number of factors, which includes glycolytic flux (Reifenberger, Freidel and Ciriacy, 1995), the presence or absence of the irreversible steps of glycolysis, glucose repression (Bisson, 1988) and catabolite inactivation (Ramos and Cirillio, 1989). A number of transporters contain consensus sites for phosphorylation by a cAMP dependent kinase (cAPK), which is known to be involved in catabolite inactivation (Bisson *et al.*, 1993). Therefore, the 'sensing' of available hexoses could be mediated by glucose transporter proteins. This would account for the need for a multigene family of glucose transporters and the complexity of the glucose response, as these transporters would be required to interact with different downstream signal transduction pathways (Bisson *et al.*, 1993). Sugar transport is also regulated by nitrogen availability and protein synthesis, such that if protein synthesis is blocked, there is an accelerated turnover of both 'low' and 'high' affinity uptake (Bustaria and Lagunas, 1986). Nitrogen limitation results in stationary phase nitrogen-depleted cells, with a decay in sugar transport activity ('stuck' fermentation), where the cessation of growth results in the elimination of glucose transporter activity.

1.3 Carbon catabolite control

Within yeast, a variety of enzyme systems are known to be repressed by externally supplied hexoses (catabolite repression). Another mechanism which also operates in the control of certain enzymes is inactivation by glucose (catabolite inactivation). Glucose also enhances the synthesis and activity of a number of enzymes, e.g. glycolytic enzymes (Paigen and Williams, 1970) and several transport systems, which include those responsible for the uptake of amino acids, inorganic ions (Borst-Pauwels, 1981; Kotyk and Michaljanicova, 1974; Ramos *et al.*, 1977), ammonia (Hemmings, 1978; Mazon and Hemmings, 1979; Roon *et al.*, 1975), inositol (Nikawa, Nagumo and Yamashita, 1982) and nucleotides (Foury and Goffeau, 1975). As all these transport systems are driven by the proton gradient, the activation of the plasma membrane proton-pumping ATPase could explain the general effect of glucose on nutrient uptake.

1.3.1 Glucose repression

Several enzyme systems in *S. cerevisiae* are subject to repression by fermentable hexoses; this repression can decrease expression by a factor greater than 100-fold for gluconeogenesis enzymes (Haarasilta and Oura, 1975) to 3-10 fold increase for mitochondrial enzymes (Polakis and Bartley, 1965; Polakis *et al.*, 1965). Numerous

mutants have been isolated which are insensitive to catabolite repression or unable to derepress in the absence of glucose. Several of these enzymes were identified by using the glucose repressed enzyme, invertase (encoded by *SUC2*) (Carlson, Osmond and Botstein, 1981; Ciriacy, 1977). Some of the principle components are described below:

A number of mutants were initially identified that were unable to grow on sucrose, even though the strains were *SUC2*+, these strains were termed Sucrose Non-Fermenting or *SNF*. *SNF1* encodes a 70 kDa serine/theorine protein kinase, which was found to be necessary for the expression of invertase (Celezna and Carlson, 1984). The protein kinase domain within Snf1p is required for its function (Celenza and Carlson, 1989). This protein kinase is present in both glucose-repressed and derepressed cells and is distributed throughout the cell (Celenza and Carlson, 1986). The *SNF1* gene product is physically associated with the *SNF4* (*CAT3*) protein, which is required for maximal kinase activity (Celenza and Carlson, 1989; Celenza, Eng and Carlson, 1989) and which has an integral function within the glucose repression signalling pathway. However, the factors which regulate this kinase activity have yet to be fully defined. *SNF1* has been reported to affect some of the same cellular responses as those directed by the cAMP dependent protein kinase (cAPK); for example, expression of invertase was reported to be regulated via both *SNF1* and a cAMP responsive protein kinase (Hubbard, Yang and Carlson, 1992).

Mutations which suppress the *snf* phenotype (termed *SSN* - Suppressor of SNF) have been identified, which allow growth of the *snf1* mutant on sucrose (Carlson *et al.*, 1984). Mutants in *SSN6* (also known as *CYC8*) (Trumbley, 1986) and *TUP1* (also known as *CYC9*) have similar properties (Williams and Trumbly, 1990). Mutations in either gene are pleiotropic producing an array of phenotypes, which include constitutive derepression of glucose repressible genes, calcium dependent flocculation, mating type defects and inability of homozygous diploids to sporulate (Williams, Varanasi and Trumbly, 1991).

The Cyc8p and Tup1p proteins are reported to be physically associated in a large complex (≈ 1,200 kDa in size) (Williams *et al.*, 1991). Although both proteins contain long stretches of polyglutamine, which are characteristic of transcriptional regulatory proteins, they possess no other similarities (Schultz and Carlson, 1987). *TUP1* and *CYC8* (*SSN6*) are involved in the repression of mating-type and the regulation of a series of genes activated or repressed by haem, e.g. *HAP1* which shows a decreased repression within a *tup1* mutant (Zhang and Guarente, 1994). The Cyc8p/Tup1p complex appears to act as global repressor of transcription. This complex is recruited to the target promoters by a variety of sequence specific DNA binding proteins, e.g.

Mig1p (Keleher *et al.*, 1992). However, the interaction between Tup1p and Cyc8p is unclear, although Cyc8p has been reported to repress transcription only when Tup1p is present. A recent report states that Tup1p is the component of the complex that causes repression and Cyc8p acts as the link to pathway specific DNA binding proteins (Tzamarias and Struhl, 1994). Functional domains within Cyc8p (the amino acid motif; threonine-proline-arginine (TPR)) are responsible for direct interaction with Tup1p and the repression of oxygen and glucose regulated genes, whereas in the Tup1p protein a tryptophan-aspartic acid (WD) motif is responsible for regulation of a distinct set of genes by DNA damage and cell-type, i.e. *MFA1* (Tzamarias and Struhl, 1995).

As neither protein possesses any recognized DNA-binding domains and as there is no evidence of this complex binding directly to the DNA, the question arises, how does this complex mediate glucose repression ?. The interaction of the Cyc8p/Tup1p complex with glucose repressed promoters is mediated through the product of the *MIG1* gene. The 56 kDa Mig1p (Multicopy Inhibitor of GAL expression) was identified as binding to two GC-rich sites within the *SUC2* promoter region. The repression of the target gene then requires the recruitment of the Cyc8p/Tup1p complex (Treitel and Carlson, 1995). Mig1p binding sites have been detected in the promoter regions of *SUC2*, *GAL1-10* and *GAL4*. however, within a *mig1* mutant, *SUC2* repression is incomplete which would suggests that additional mechanisms of repression are also active (Nehlin and Ronne, 1990). It also appears that the Cyc8p/Tup1p complex can act both as a repressor and inhibitor of *MIG1*. These observations suggest that the *CYC8* TPR motif and *TUP1* WD motif mediate different protein-protein interactions, which link the Cyc8p/Tup1p corepressor to various DNA binding proteins required for specific pathway regulations (Tzamarias and Struhl, 1995).

From the epistatic relationships between the identified regulatory mutants, several pathways of glucose repression have been proposed (Donnini *et al.*, 1990; Gancedo, 1992; Trumbley, 1992). The pathway suggested by (Trumbley, 1992) (figure 1.2) involves the bifunctional enzyme hexokinase P-II (*HXK2*), which possesses both catalytic and regulatory activity (Entian and Frohlich, 1984; Entian *et al.*, 1984), as the primary element in the 'sensing' of external glucose (Niederacher and Entian, 1987; Niederacher and Entian, 1991). Constitutive expression of glucose repressible genes has been reported in the mutants *grr1* (*cat80*) (Flick and Johnston, 1991; Vallier and Carlson, 1991), *hxk2* (Entian and Frohlich, 1984) and *reg1* (*hex2*) (Niederacher and Entian, 1987; Niederacher and Entian, 1991), which are not blocked by *SNF1/SNF4* and are therefore positioned before *CYC8/TUP1* in the regulatory

pathway (figure 1.2). The Cyc8p/Tup1p transcriptional regulation complex is then targeted via gene specific DNA binding proteins, e.g. Mig1p to the target gene, thus resulting in repression. The release from glucose repression is brought about by the action of Snf1p on the Cyc8p/Tup1p/Mig1p complex (Trumbley, 1992).

1.3.2 Regulation by cyclic AMP dependent protein kinase phosphorylation

The physiological status of the yeast cell is reflected by the intracellular cAMP level, which varies in response to changes in the external glucose concentration. Endogenous cAMP levels decline upon entry into the stationary phase, when cell proliferation is arrested (Mazon, Gancedo and Gancedo, 1982), which has also been correlated with developmental changes within the yeast cell; for example a decrease in cAMP concentration is correlated to the induction of certain stress response proteins (Boorstein and Craig, 1990a; Fraser *et al.*, 1991; Praekelt and Meacock, 1990; Shin *et al.*, 1987) and the 'high' affinity uptake of glucose (Ramos and Cirillio, 1989). The activities of certain enzymes, e.g. glycogen phosphorylase and trehalase (which are necessary for the mobilization of reserve carbon sources) are regulated by a phosphorylation-dephosphorylation mechanism (Matsumoto, Uno and Ishikawa, 1985).

The effects of cAMP on cellular processes are mediated by a cAMP dependent protein kinase in the RAS/cAMP pathway (reviewed in (Broach, 1991)). The first element in this pathway is encoded by the gene, *CDC25*, which is directly involved in 'sensing' the physiological status of the cell (Munder and Kuntzel, 1989). Signals from the Cdc25p are transmitted via the RAS proteins to adenylate cyclase (encoded by *CYR1*) which converts ATP to cAMP (Matsumoto *et al.*, 1985) and the high affinity cAMP phosphodiesterase (encoded by *PDE1* and *PDE2*) (Sass *et al.*, 1986). In turn, cAMP regulates the activity of a cAMP dependent protein kinase composed of three catalytic sub-units (encoded by *TPK1*, *TPK2* and *TPK3*), complexed with a regulatory sub-unit encoded by *BCY1* (Cameron *et al.*, 1988). The cAMP binds to the Bcy1p regulatory sub-unit, which then dissociates to release an active kinase complex, which phosphorylates targeted proteins (figure 1.2). Consistent with this, cells lacking the regulatory Bcy1p sub-unit do not respond to nutrient limitation and *cyr1* mutants show a constitutive protein kinase activity (Matsumoto *et al.*, 1985).

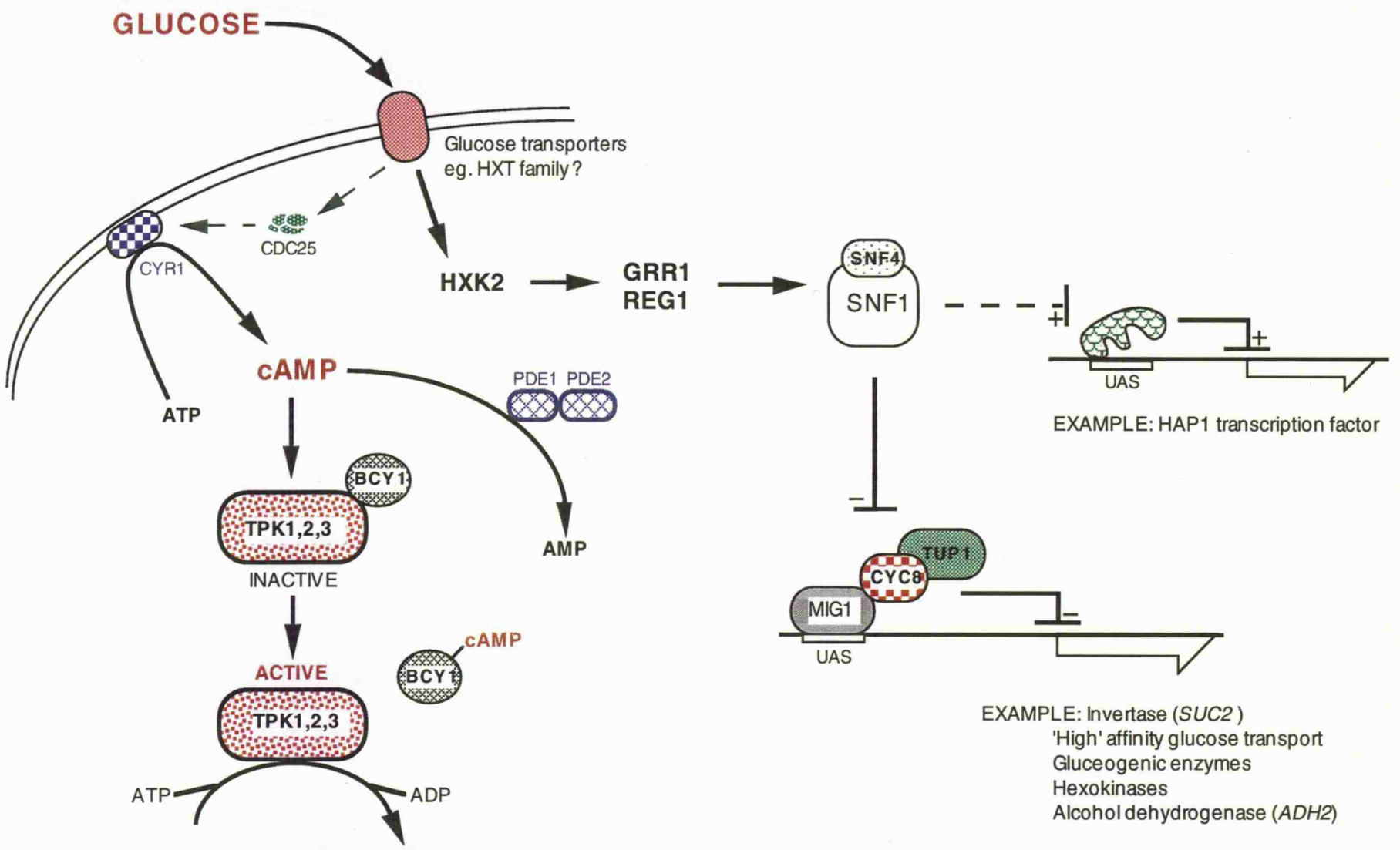


Figure 1.2: Epistatic relationships between catabolite mutants in *Saccharomyces cerevisiae*. Mutations are as indicated in text. Dashed lines indicate a possible relationship between two mutants. Model is based upon that proposed by Trumbley (1992). cAMP pathway is based upon the model proposed by Broach (1991).

1.4 Stress response in *S. cerevisiae*

Starvation is probably the most common stress challenge endured by the yeast cell, however, other stresses can also be encountered e.g. lethal temperatures, osmotic shock and changes in environmental pH. In response to these challenges yeast cells display a rapid molecular response (reviewed in (Mager and Moradas-Ferreira, 1993; Piper, 1993)). The most striking feature is the synthesis of a set of proteins, including the so-called 'heat shock proteins'. In *S. cerevisiae*, a sudden temperature change results in a dramatic, temporary alteration in the pattern of protein synthesis (McAlister *et al.*, 1979; Miller, Xuong and Geidusche, 1979). This ability of the cell to shift rapidly to heat shock protein synthesis suggests a pivotal role in the cell survival mechanism. Heat shock proteins in *S. cerevisiae* are synthesized in response to a stress challenge and are distinguished according to their average apparent molecular masses e.g. *HSP12* (Praekelt and Meacock, 1990), *HSP26* (McAlister *et al.*, 1979; Petko and Lindquist, 1986), *HSP30* (Regnacq and Boucherie, 1993), the *HSP70* family (Werner-Washburne *et al.*, 1989), *HSP83* (Borkovich *et al.*, 1989) and *HSP104* (Sanchez and Lindquist, 1990).

This heat shock response is an inducible protective mechanism, which results in the synthesis of heat shock proteins, and the ability to the cell to withstand potentially lethal stresses i.e. high temperature, high osmolarities and other agents. The functional relevance of individual heat shock proteins is not fully understood and only a minority seem to be required for thermotolerance i.e. Hsp104p, Ssa3p (*HSP70* family), whereas some heat shock proteins i.e. Hsp60p, have been shown to act as molecular chaperones (Hightower, 1991). However, heat shock proteins have been implicated in all major growth processes i.e. cell division, DNA synthesis, transcription, translation, membrane function, protein folding and transportation.

In *S. cerevisiae*, the *HSP70* multi-gene family, consists of eight members, sub-divided into four sub-families; *SSA*, *SSB*, *SSC* and *SSD*, which show an amino acid sequence identity between 50% to 97%. Expression of the individual family members is regulated differently in response to changes in growth conditions. The *SSA* family is essential for growth and encodes for the Ssa1p, Ssa2p, Ssa3p and Ssa4p proteins which are all localised in the cytoplasm (Boorstein and Craig, 1990b; Slater and Craig, 1987). *SSA3* is unique that it is only expressed at extremely low levels under optimal conditions, but is rapidly induced by entry into the diauxic lag or upon heat shock (Werner-Washburne, Stone and Craig, 1987). *SSA4* is a characteristic heat shock gene, which displays a low basal level of expression and is strongly induced

upon heat treatment. In contrast, *SSA1* and *SSA2* show a constitutive expression. Mutations in either *SSB1* and *SSB2* result in a cold sensitive phenotype; the cellular localisation of their protein products is thought to be cytoplasmic and associated with translating ribosomes (Boorstein, Ziegelhoffer and Craig, 1994; Nelson *et al.*, 1992). Both *SSC1* and *SSD1* are essential for growth, where the Ssc1p is a mitochondrial protein (Craig, Kramer and Kosicsmithers, 1987; Craig *et al.*, 1989) and Ssd1p (also known as Kar2p) is found within the endoplasmic reticulum (Polaina and Conde, 1982). The Hsp70 proteins contain an ATP binding site, with a weak ATPase activity (Chappell *et al.*, 1987) and play a role in aiding protein-protein interactions. These interactions have been postulated to involve the binding to certain polypeptide chains, hence modifying or maintaining their conformation. Under normal conditions, Hsp70p's were found to facilitate the translocation of polypeptides across the endoplasmic reticulum and mitochondrial membranes (Chirico, Waters and Blodel, 1988; Deshaies *et al.*, 1988). Yeast cells lacking *SSA1* and *SSA2* appear to accumulate precursor forms of proteins, normally destined for import into the endoplasmic reticulum, thus indicating that Ssa1p and Ssa2p are involved in post-translational import pathways. *SSD1* (*KAR2*) is a homologue to the mammalian BiP protein, which aids transport of glycoslylated proteins through the secretory pathways from the endoplasmic reticulum to the golgi body (reviewed in (Deshaies *et al.*, 1988; Pelham, 1989)). All the *SSA*, *SSC* and *SSD* sub-family genes encode proteins that act as molecular chaperones. It is likely that hsp70s are induced under stress, to perform functions similar to the cognate proteins present under normal conditions. During stress, the cellular concentration of aberrant proteins increases, thus depleting the free pool of Hsp70p, which in-turn generates the need for an increase in the levels of these proteins.

HSP60 encodes a mitochondrial protein, which facilitates the post-translational assembly of polypeptides and hence is a molecular chaperone (Ellis, 1987; Ellis and Vandervies, 1991). Proteins imported into the mitochondria, require the Hsp60p for proper folding (Koll *et al.*, 1992; Neupert *et al.*, 1990; Ostermann *et al.*, 1989). In *S. cerevisiae*, *HSP83* and *HSC83* (representative of the family of Hsp90 proteins) encode chaperone-like proteins, which also act in the signal transduction pathways for steroid receptors. *HSC83* is a constitutively expressed gene, which is only expressed weakly upon stress exposure. In contrast, *HSP83* is expressed at a much lower basal level, but is strongly induced after heat shock. Expression of this gene is also induced when cells enter into stationary phase or sporulate (Kurtz and Lindquist, 1984; Kurtz *et al.*, 1986). Corresponding homologues to yeast Hsp90's in mammalian cells have been found to interact with a variety of cellular proteins, including glucocorticoid receptors, several kinases and the cytoskeleton proteins, actin and tubulin. The

functions encoded by heat shock proteins with a molecular mass greater than 100 kDa are yet to be defined. In yeast, Hsp104p is not detectable during normal growth on fermentable carbon sources, but is expressed constitutively in respiring cells and is strongly induced upon heat shock (Sanchez *et al.*, 1992). Expression of this gene is induced by entry into stationary phase or by sporulation of the yeast cell and is essential for long term survival under nutrient depleted conditions (Sanchez*et al.*, 1992). Two putative nucleotide consensus binding sites have been identified in Hsp104p, which are essential for this stress protection function (Parsell *et al.*, 1991).

Within this study, a discussion of the 'small' heat shock proteins, *HSP12*, *HSP26* and *HSP30* is pertinent. These small heat shock proteins are characterized by their high level of induction either by stress or progression through the growth cycle (Susek and Lindquist, 1989). Induction of these genes can be initiated by any of the following stress regimes e.g. ethanol (Piper *et al.*, 1994; Plesset, Palm and McLaughlin, 1982), methanol (Kirk and Piper, 1991), DNA damage agents (McClanahan and McEntee, 1986), glucose limitation (Bataille, Regnacq and Boucherie, 1991), temperature increase (Pelham, 1985) and osmotic stress (Varela *et al.*, 1992). However, the functions performed by these small heat proteins is not known (Tuite *et al.*, 1990).

The *HSP26* gene has been characterized and is reported to play no vital role in the heat shock response (Bossier *et al.*, 1989; Petko and Lindquist, 1986; Tuite *et al.*, 1990). *HSP26* expression increases during normal development of the culture as it enters stationary phase (Susek and Lindquist, 1990) or when subject to nutrient limitation (carbon limitation) to induce sporulation (Kurtz and Lindquist, 1984). The intracellular distribution of Hsp26p is dependent upon the physiological state of the cell, where after heat shock it is concentrated within the cell nucleus (Rossi and Lindquist, 1989). When Hsp26p is over-expressed, an aggregated product (heat shock granules, HSG's) is formed as high molecular weight complexes or filaments (Bentley, Fitch and Tuite, 1992). The primary peptide sequence of Hsp26p contains the amino acid motif; *G-[V/I]-L-T-[X]$_3$-P* consensus sequence in the carboxyl terminus, which is similar to that in the cytoskeletal protein actin (residues 61-69). These observations suggest that the aggregated complexes are involved in maintaining cell infrastructure, by possible stabilization of actin filaments (Rahman, Bentley and Tuite, 1995). Transcriptional regulation of *HSP26* is via both a *cis*-regulatory element, which appears to moderate heat shock transcription and a separate mechanism which regulates developmental expression, although this element remains to be identified (Susek and Lindquist, 1990). Within the *HSP26* promoter, multiple copies of the stress responsive element (STRE) are also found, which maybe the

site(s) for the developmental regulation exhibited by this gene. The rate of induction of the *HSP26* has been shown to be dependent upon the heat shock factor occupancy of the heat shock element, rather than the number of elements within the promoter (Chen and Pederson, 1993). *HSP26* expression has been reported to be unaffected by changes in cAMP levels (Silva *et al.*, 1994).

HSP12 is induced by both heat shock and entry into stationary phase, but is not essential for cell survival (Praekelt and Meacock, 1990). Induction of *HSP12* is distinct from that of *HSP26*, in that a decline in intracellular cAMP concentration, results in the expression of *HSP12*. A similar response was reported for the large heat shock gene, *HSP70* (*SSA3*) (Boorstein and Craig, 1990a; Cameron *et al.*, 1988; Tanaka *et al.*, 1989). Both *HSP12* and *HSP26* have been reported to be regulated via Msn2p and Msn4p; cys_2his_2 zinc-finger proteins similar to Mig1p (see section 1.3) (Martinez-Pastor and Estruch, 1995). The remaining identified small heat shock protein in *Saccharomyces cerevisiae* is *HSP30*, which appears to be localised to the plasma membrane (Panaretou and Piper, 1992). *HSP30* is induced by both ethanol stress and heat shock, its function appears to be involved in the maintenance of the electrochemical gradient across the cell membrane, in times of stress.

There is a strong similarity between the cellular response to ethanol and to those observed after heat shock. Ethanol accumulates in batch fermentation, where it reduces the specific growth rate, the fermentation rate and cell viability (van Uden, 1984). The adverse effects of ethanol on the cell, involves the alteration in membrane associated processes. The forms of Hsp90p and Hsp70p induced by ethanol are the same as those induced by heat. These two stresses also effect the levels of two major plasma-membrane proteins; ATPase and Hsp30p (Piper *et al.*, 1994). There is a critical threshold at which heat shock proteins are induced by ethanol, where a rise in ethanol levels from 4 to 10% increases the synthesis of Hsp104p, Hsp70p and Hsp26p. Both Hsp30p and Hsp12p levels decline as ethanol levels increase above 6%. This suggests that the level of induction of different heat shock genes is regulated by factor(s) that sense different sub-lethal ethanol concentrations.

Genes other than those of heat shock proteins are also induced by a variety of stress conditions or entry into stationary phase; these include those involved in trehalose degradation (Francois *et al.*, 1991; Panek and Panek, 1990; Wiemken, 1990), *CTT1* and *UBI4*. *UBI4* is a heat -inducible (Ozkaynak *et al.*, 1987) and stress responsive gene (Fraser *et al.*, 1991), which is involved in the essential removal of toxic aberrant or damaged proteins from the cell. As stress leads to a sudden increase in aberrant proteins, *UBI4* transcription increases (Grant, Firoozan and Tuite, 1989) and hence ubiquitin levels increase to target these proteins for degradation. *ubi4*

mutants contain normal concentrations of free ubiquitin, however, these mutants are hyper-sensitive to high temperatures and other stresses e.g. starvation. Within the *UBI4* promoter region, consensus sequences to the heat shock element (see below) have been identified, which would account for *UBI4* heat shock induction (Ozkaynak *et al.*, 1987). *UBI4* is essential for long term cell survival under nutrient limitation (Finley *et al.*, 1987)

Cytosolic catalase T1 (encoded by *CTT1*), may contribute in the protection against the adverse affects of heat shock and whose rate of synthesis is controlled by heat shock (Belazzi *et al.*, 1991), and in addition several enzymes in the glycolytic pathway are also induced by heat shock i.e. glyceraldehyde-3-phosphate dehydrogenase (Lindquist and Craig, 1988), enolase (Iida and Yahara, 1985) and phosphoglycerate kinase (Piper *et al.*, 1988). The increased synthesis of these enzymes maybe beneficial, as the cell is able to increase the rate of glycolysis and restore intracellular cAMP or that stress damage has altered membrane structures, thus disrupting the normal coupling between electron transport and oxidative phosphorylation (Patriarca and Maresca, 1990). During stress intracellular pH drops, due to the dissipation of the electrochemical pH gradient across the plasma membrane. This change in intracellular pH may play a direct or indirect role in triggering the stress response (Coote, Cole and Jones, 1991). The plasma membrane ATPase (encoded by *PMA1*), maintains this pH gradient and upon heat shock, expression of the *PMA1* is unaffected (Panaretou and Piper, 1990).

A decline in intracellular cAMP, associated with entry into stationary phase and probably the subsequent decrease in cAPK activity, triggers the synthesis of several heat shock proteins (Iida and Yahara, 1984; Shin *et al.*, 1987). With a decline in cAMP levels, *SSA1* (Brazzell and Ingolia, 1984), *HSP12* (Praekelt and Meacock, 1990) and *UBI4* (Fraser *et al.*, 1991) all show an enhanced expression. Thus, a RAS-adenylate cAMP dependent phosphorylation mechanism plays an important role in the control and induction of certain stationary phase genes (Piper, 1990). However, the cAMP pathway does not initiate the heat shock response (Shin *et al.*, 1987), as heat shocked cells contain elevated levels of intracellular cAMP, therefore the heat shock response is not mediated via the same signal pathway as the cAMP dependent regulation (reviewed in (Piper, 1990)). The effect of cAMP on heat shock gene expression is not mediated through the heat shock element (HSE) (see below), as a HSE-*Lac*Z construct was found to give no cAMP dependent or growth phase dependent expression (Piper, 1990).

The different expression patterns reported for the heat shock proteins suggest that a variety of regulatory mechanisms act upon their expression. Two conserved regions

have been identified, within the various heat shock gene promoters; the first is the heat shock element (HSE) i.e. 5' CnnGAAnnTTCnnG 3' (Sorger and Pelham, 1987), found upstream of the TATA box within heat shock promoters (Tuite, Bossier and Fitch, 1988). Upon heat shock, a pre-existing pool of trimeric *trans*-acting transcriptional activators (HSTF) bound to the HSE are converted to an active form, which then stimulates transcription. As phosphorylation of this heat shock factor creates a complex with increased transcriptional activity, dephosphorylation maybe involved in the control of these genes (Sorger, 1990). A temperature-regulated transcriptional activation domain within the HSTF, is essential for survival of cells after heat shock and maybe the site of action for a derepression type of control in the heat shock response (Nieto-Sotelo *et al.*, 1990).

The second element that has been identified in certain heat shock gene promoters, is the stress response element (STRE), initially identified in *CTT1* (Bissinger *et al.*, 1989), which directs induction by nitrogen starvation or heat shock (Belazzi *et al.*, 1991; Marchler *et al.*, 1992). One example of this type of signal pathway is the *HOG1/MAP* kinase pathway which mediates the response to osmotic stress (Schuller *et al.*, 1994). The *HOG1/MAP* kinase pathway is defined by the *HOG1* and *PBS2* genes, which encode members of the MAP kinase and MAP kinase kinase family, respectively (Schuller *et al.*, 1994). An STRE mediated induction of the stress proteins, *HSP12*, *HSP104* and *CTT1* by heat shock, nitrogen starvation and oxidative stress has been reported via a *HOG1* dependent pathway. However, low external pH and ethanol stress appears to induce expression via a *HOG1* independent mechanism (Schuller *et al.*, 1994). The osmotic stress response of the heat shock gene *HSP12* is thought to be mediated via an STRE (Varela *et al.*, 1995; Varela *et al.*, 1992). A possible STRE in the *HSP12* promoter, consisting of five repeats of the motif, CCCCT present in both orientations, in which a wide redundancy in activation or repression appears to operate has been identified. This element has also found within the *HSP26* gene promoter (at position -22 nt 5' to the translation ATG start site), however, the significance of this element at this location has not been assessed.

1.5 Thiamine biosynthesis, transport and control

In the context of this thesis a discussion on the transportation and biosynthesis of the vitamin thiamine is relevant. In *S. cerevisiae*, thiamine is a precursor of the important enzyme cofactor, thiamine pyrophosphate, which is required by several glycolytic enzymes e.g. pyruvate decarboxylase. Thiamine pyrophosphate serves as a coenzyme, by acting as a transient intermediate carrier of an activated aldehyde group. Thiamine is present in the external cell environment as the free form or more

usually as the phosphate, both of which are rapidly assimilated by the cell, in preference to its *de novo* synthesis.

Thiamine uptake has yet to be fully described, however, it is known to be an energy dependent process, where accumulated thiamine exists intracellulary in the free form (Iwashima, Nishino and Nose, 1973). In *S. cerevisiae*, extracellular thiamine is rapidly sequestered from the external environment by a thiamine regulated process to an intracellular concentration of 1,600 pmoles/10^7 cells (Praekelt, Byrne and Meacock, 1994). One identified component in this transport mechanism is a thiamine repressible acid phosphatase, Pho3p (Bajwa, 1984). Pho3p is a 56KDa thiamine-binding acid phosphatase (T-rAPase), localised in the periplasmic space of the yeast cell wall (Iwashima and Nishimura, 1979; Schweingruber *et al.*, 1986), which requires two complementary genes *PHO6* and *PHO7* for it's regulation and synthesis, respectively (Nosaka *et al.*, 1992; Toh-e, Kakimoto and Oshima, 1975). The Pho3p acid phosphatase exhibits a high affinity for thiamine phosphates and catalyses their hydrolysis within the periplasmic space, prior to transportation of the thiamine (free form) into the cell (Nosaka, 1990; Nosaka *et al.*, 1989). Thiamine binding proteins which are present in the plasma membrane are thought to participate directly in thiamine transport (Iwashima and Nishimura, 1979; Iwashima, Nishimura and Nose, 1979). The identified precursors to thiamine, 2-methyl-4-amino-5-hydroxymethyl-pyrimidine and 4-methyl-5-ß-hydroxyethylthiazole are rapidly taken up to form thiamine by resting yeast cells. The pyrimidine precursor is taken up into the yeast cell via an energy-dependent thiamine transport mechanism. In contrast to thiamine, excess accumulated hydroxymethylpyrimidine is released (Iwashima, Kawasaki and Kimura, 1990). The hydroxyethylthiazole precursor is transported into yeast cells by diffusion, followed by metabolic trapping by hydroxyethyl thiazole kinase-catalysed phosphorylation (Iwashima *et al.*, 1986).

The enzymatic steps leading from the precursors, 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-ß-hydroxyethylthiazole to the *de novo* synthesis of thiamine by the yeast cell have been identified. Synthesis of the thiamine moiety involves the phosphorylation and condensation of these moieties to form the monophosphate (figure 1.3) (White and Spenser, 1979; Young, 1986). However, the biosynthetic pathways leading to the formation of these precursors has yet to be fully elucidated. It is clear that neither uracil or its precursors are utilized in the formation of the pyrimidine moiety, therefore, another pathway of pyrimidine synthesis must exit. One proposed pathway is thought to involve pyridoxine as an intermediate (Tazuya, 1993; Tazuya *et al.*, 1994), where the nitrogen atom and part of the carbon backbone of pyridoxine is derived from the amino acid, glutamine (Tazuya *et al.*,

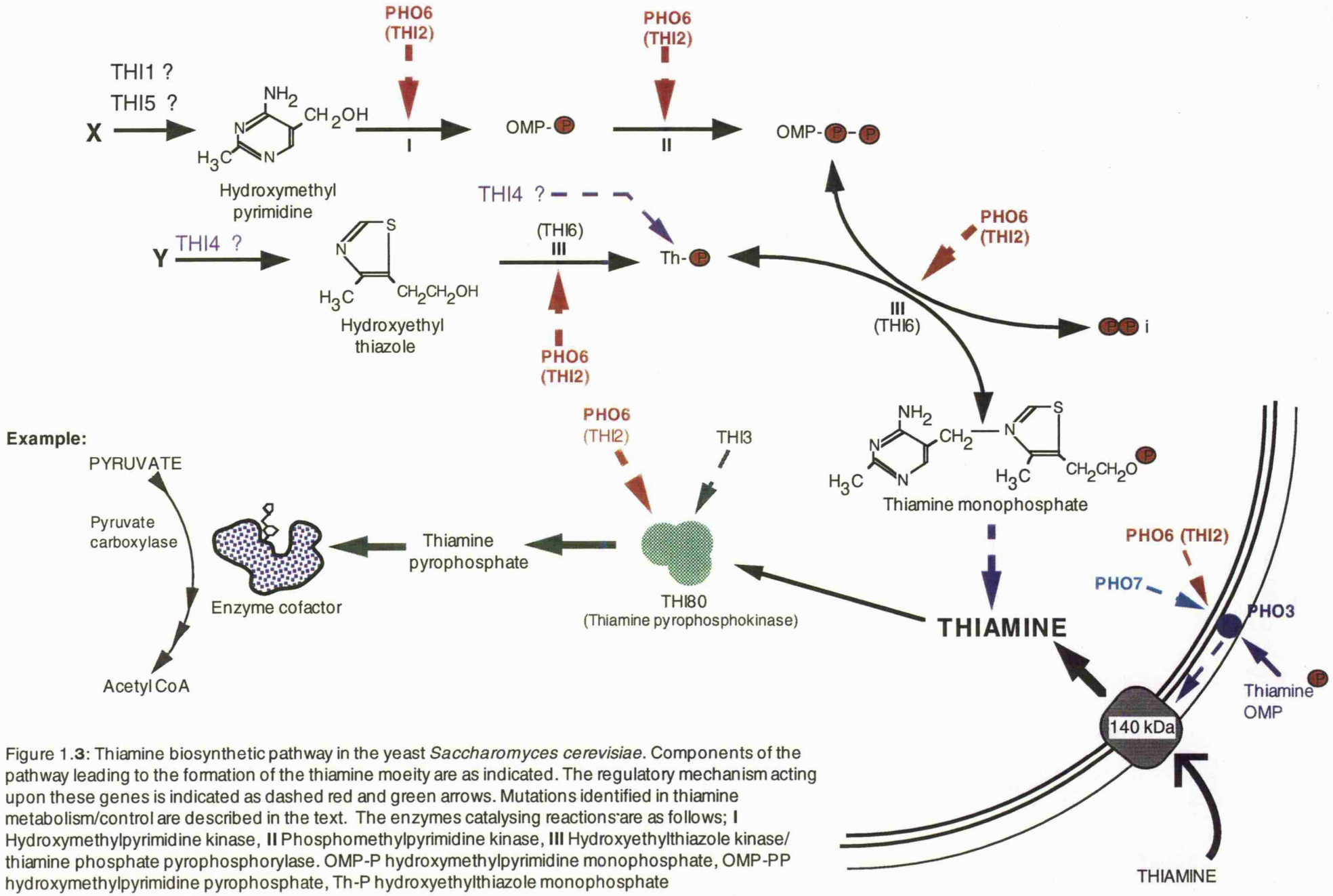


Figure 1.3: Thiamine biosynthetic pathway in the yeast *Saccharomyces cerevisiae*. Components of the pathway leading to the formation of the thiamine moeity are as indicated. The regulatory mechanism acting upon these genes is indicated as dashed red and green arrows. Mutations identified in thiamine metabolism/control are described in the text. The enzymes catalysing reactions are as follows; **I** Hydroxymethylpyrimidine kinase, **II** Phosphomethylpyrimidine kinase, **III** Hydroxyethylthiazole kinase/ thiamine phosphate pyrophosphorylase. OMP-P hydroxymethylpyrimidine monophosphate, OMP-PP hydroxymethylpyrimidine pyrophosphate, Th-P hydroxyethylthiazole monophosphate

1995). The thiazole moiety has been reported to be synthesized from a pentose intermediate, which is generated from hexose precursors via the pentose phosphate pathway and condensed with the amino acid, glycine (White and Spenser, 1979). The condensation reaction of the phosphorylated thiazole to the pyrimidine precursor to form thiamine monophosphate is catalysed by the bifunctional enzyme, thiamine phosphate pyrophosphorylase / hydroxyethylthiazole kinase (encoded by *THI6*).

Both the *de novo* synthesized or the externally derived thiamine is converted to the important gluceogenic enzyme cofactor, thiamine pyrophosphate by thiamine pyrophosphokinase (encoded by *THI80*) (Nishimura *et al.*, 1991). *THI80* is essential for cell survival and shows a constitutive low level of expression that is not completely repressed by exogenous thiamine (Nosaka *et al.*, 1993). A *thi80* mutant exhibits a marked alteration in the activities of the thiamine repressible acid phosphatase (*PHO3*), the thiamine transport system and identified enzymes involved in thiamine synthesis between 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-ß-hydroxyethylthiazole. These observations suggest that utilization of thiamine in *S. cerevisiae* is controlled negatively by the level of intracellular thiamine pyrophosphate (Nishimura *et al.*, 1991). Within this thesis two more elements were characterized within the thiamine biosynthetic pathway; the previously reported gene *THI4* (Praekelt *et al.*, 1994) and *THI5* (Hather, Praekelt and Meacock, In preparation). These two genes were identified as being involved in the thiazole and pyrimidine biosynthetic pathways, respectively.

Regulation of thiamine metabolism in *S. cerevisiae* appears to involve two positively acting genes; *PHO6* (*THI2*) (Nishimura *et al.*, 1992a) and *THI3* (Nishimura *et al.*, 1992b). A *pho6* (*thi2*) mutant was found to be auxotrophic for thiamine and the activities of the four identified enzymes involved in the synthesis of thiamine monophosphate were hardly detectable. This indicated that thiamine synthesis is subject to the positive regulator *PHO6*, which is in turn controlled by the intracellular thiamine level (Kawasaki *et al.*, 1990). It is thought that the *THI3* gene is involved in a regulatory mechanism distinct from that mediated via *PHO6* (Nishimura *et al.*, 1992b), as in a *thi3* mutant a marked reduction in the level of thiamine transport and the activity of several biosynthetic enzymes in both the pyrimidine and thiazole precursor pathways is observed. Therefore, the positive regulatory gene *THI3* is involved in the control of both thiamine transport (including the *PHO3* acid phosphatase) and metabolism (Nishimura *et al.*, 1992b). Thiamine pyrophosphokinase (*THI80*) has been reported to be controlled via *PHO6* (*THI2*) and *THI3* (Nosaka *et al.*, 1993).

In the yeast *Schizosaccharomyces pombe* several thiamine regulated genes have been identified; *pho4* (a thiamine regulated acid phosphatase) (Schweingruber *et al.*, 1986, *nmt1a* (*thi3*) (Maundrell, 1990; Yang and Schweingruber, 1990), *thi1* (Fankhauser and Schweingruber, 1994), *thi2(nmt2)* (Manetti, Rosetto and Maundrell, 1994) and *thi4* (Schweingruber *et al.*, 1991). Both *nmt1a* and *thi4* are reported to be involved in the synthesis of the pyrimidine precursor, with *thi2* (*nmt2*) involved in the synthesis pathway leading to the thiazole precursor. The *thi1(ntf1+)* gene appears to encode a 775 amino acid protein with the characteristics of a Cys_6 zinc-finger motif transcription factor, which binds to upstream activator sequences of thiamine repressible genes (Fankhauser and Schweingruber, 1994). Within *S. pombe*, thiamine was shown to inhibit sexual agglutination and zygote formation at a concentration of 50mM and 1μM, respectively (Schweingruber and Edenharter, 1990). Within *S. pombe* extracellular thiamine is rapidly sequested from the external environment by a thiamine regulated process to an intracellular concentration of 9,000 pmoles/10^7 cells, which is significantly higher than that reported in *S. cerevisiae* by (Praekelt *et al.*, 1994). Transcription of *nmt1a* is repressed by an internal thiamine concentration above 50 pmoles/10^7 cells (Tommasino and Maundrell, 1991). A similar intracellular thiamine concentration has been reported to repress the transcription of the *S. cerevisiae* homologue of *nmt2*, *THI4* (Praekelt *et al.*, 1994). Expression of *nmt1a* has been reported to be regulated via the *ntf1+* transcription factor, where deletion of the *ntf1+* binding site within the *nmt1a* promoter region results in a decrease in the basal level of gene expression (Tang, Bueno and Russell, 1994).

1.6 Aims

A series of stationary phase induced genes were isolated from cultures of the yeast *S. cerevisiae* grown on molasses medium (Praekelt, U. and P. A. Meacock, 1990). The aim of this study was to characterize this series of genes, starting from their respective cDNA clones, in order to elucidate their function and control mechanisms. The initial rational for the isolation of these genes was that since they are induced at the end of fermentative growth and on entry into stationary phase the promoters maybe useful for regulated expression of heterologous proteins. By coupling a foreign gene to such a promoter the protein could be produced naturally during the growth cycle after the culture biomass had accumulated. Before these promoters can be utilized industrially, it would be important to understand what genetic controls were operating upon them and under what conditions they are induced. An outline of this characterization is as follows:

i) Examination of expression under a variety of growth conditions and in numerous mutant strains, to assess possible control mechanisms.

ii) Sequence analysis of the cDNA clone and comparison to the EMBL database to identify each clone.

iii) Recovery of genomic clones for the characterized genes that appear to be novel.

Chapter 2:
Gene expression patterns on different growth media

2.1 Introduction

2.1.1 Characteristics of aerobic batch cultures

S. cerevisiae exhibits several different stages of growth (Lewis *et al.*, 1993) dependent upon carbon source and physiochemical factors within the external environment (Berry and Brown, 1987). An aerobic batch culture of *S. cerevisiae* on glucose shows six phases of growth i.e. initial lag, respiro-fermentative, diauxic lag, respiratory, stationary and death phase (Lewis *et al.*, 1993). During the respiro-fermentative phase, where glucose concentration is initially high, growth is rapid and is characterised by a specific growth rate (μ) of 0.40-0.45 hr^{-1}, accompanied by the production of ethanol, a low biomass yield and a high respiratory quotient (ratio of CO_2 produced to O_2 consumption rate) (Lievense and Lim, 1982). Upon glucose depletion, a lag period of growth is observed (the diauxic lag), during which a series of adaptive changes occur allowing the use of alternative carbon sources (the shift from fermentative to respiratory growth). A second period of respiratory growth on ethanol then occurs, distinguishable by a lower specific growth rate (μ) 0.14-0.20 hr^{-1}, a higher biomass yield and low respiratory quotient (characteristic of oxidative metabolism of ethanol) (Fiechter *et al.*, 1987).

Cells in the stationary phase of growth are characterized by cessation of cell proliferation (Drebot *et al.*, 1990) and the acquisition of a series of distinct physiological properties i.e. increased thermotolerance (Shin *et al.*, 1987), mobilisation and depletion of storage carbohydrates e.g. glycogen and trehalose (Lillie and Pringle, 1980). Both glycogen and trehalose metabolism responds to a glucose repression control, where upon glucose depletion, glycogen is hydrolysed preferentially to trehalose (Lillie and Pringle, 1980). Trehalose has been reported to aid in the protection of yeast cells against stationary phase starvation and against a variety of stresses i.e. osmotic and heat shock (Wiemken, 1990).

2.1.2 Isolation of differentially expressed genes from *S. cerevisiae*

A series of cDNA clones representing 'stationary phase' induced genes were isolated by differential mRNA hybridization from molasses-grown *S. cerevisiae* cells by Dr U. Praekelt (Praekelt and Meacock, 1990). The isolation of stationary phase induced genes by a differential screen depends on clear differences between the

mRNA populations of exponential and near stationary phase cells. *In vivo* labelled proteins of yeast cells show a marked increase in the synthesis of several proteins on entry into stationary phase (Boucherie, 1985), suggesting that cDNA probes based on the two mRNA populations should indeed be suitable as differential hybridization probes. Although the ultimate goal is the isolation of complete genes with their regulatory promoter elements, initially a cDNA library of stationary phase RNA was screened, since this should be highly enriched in the coding sequences of interest.

RNA was prepared from yeast cells of strain S288C grown in molasses medium and harvested just after the cessation of exponential growth, during transition to stationary phase. cDNA was synthesized using the poly(A) fraction of RNA and cloned via *Eco*RI linkers into the *E. coli* bacteriophage vector, λgt10. A library of approximately 10,000 recombinants was screened by differential plaque hybridization, using radioactive first strand cDNA synthesized from RNA populations isolated from exponentially growing and near stationary phase cells. An initial 172 clones selected in this way were then subjected to a second round of screening. Of these, 92 clones again showed differential hybridization. The complexity of the clones was established by sequential cross-hybridization, using cDNA inserts from randomly selected clones as probes. All of the 92 differentially expressed sequences were assigned to only six different hybridization classes, where clones within each hybridization class showed comparable levels of hybridization with the differential probe.

Of the six classes of clones identified, the sizes of the cognate mRNA were used to calculate the potential size of the proteins encoded by these genes. Two clones were identified by partial DNA sequence analysis as the previously reported genes *HXK1* (encoding hexokinase A) (Entian *et al.*, 1984; Kopetzki, Entian and Mecke, 1985) and *HSP26* (Bossier *et al.*, 1989) (encoding the small heat shock protein Hsp26p). A third class was identified as a new heat shock gene, *HSP12* (Praekelt and Meacock, 1990). A fourth cDNA clone, *ESP35* was characterized as being capable of coding for a polypeptide of 35 kDa and has now been identified as the thiamine regulated gene; *THI4* (*MOL1*) (Praekelt and Meacock, 1992; Praekelt *et al.*, 1994). A fifth cDNA clone termed, *ESP65* represents a gene encoding a polypeptide of 65 kDa. The sixth cDNA represents a gene capable of encoding a polypeptide of 30 kDa and was termed *ESP30*. Characterization of these latter two genes, *ESP65* and *ESP30*, is the subject of the latter parts of this study.

The six classes of stationary phase induced genes were initially identified in *S. cerevisiae* cells cultured on blackstrap molasses (a by-product of the sugar extraction process from sugar cane). Blackstrap molasses is utilised as a cheap substrate in industrial yeast fermentation (Casida, 1968; DuToit, Oliver and Van Biljon, 1984). The mother liquor contains approximately 52% (w/v) total sugars, calculated as 30% (w/v) sucrose and 22% (w/v) invert sugars (a mixture of glucose and fructose), with varying raffinose concentrations (DeWhalley, Albon and Gross, 1951). Small amounts of complex polysaccharides, with various non-carbohydrate compounds, i.e. nitrogen containing polymorphic substances and high concentrations of inorganic ions/organic acids are also present, e.g. aconitic, malic, citric, lactic, formic, acetic and propionic acids. The main amino acids found include asparagine and glutamine. Also present are heat stable vitamins (i.e. myo-inositol, niacin, pantothenic acid, riboflavin and biotin) and organic phosphate compounds e.g. inositol hexaphosphate. Molasses media has a polymorphic nature with an overall composition dependent on specific areas of geographic production, particularly for metal ion content.

In this study, the expression profiles for the six genes was initially examined in yeast cells grown on blackstrap molasses medium, in order to confirm the original isolation patterns (Praekelt and Meacock, 1990). Characterization of expression was then undertaken on more defined media, supplemented with a variety of single carbon sources and under controlled environmental conditions, the rationale being that comparison of expression patterns under defined growth conditions would aid in the identification of functions encoded by these genes and the regulation mechanisms acting upon them.

2.2 Gene expression in yeast grown on blackstrap molasses

The pattern of protein expression between respiro-fermentative and stationary phase cells is dramatically different (Boucherie, 1985), where induction of heat shock proteins accounts for one of the major changes in protein expression (Bataille *et al.*, 1991). In order to assess that yeast cells grown on blackstrap molasses do exhibit a change in the pattern of gene expression through the different growth phases, changes in the pattern of *de novo* protein synthesis at various points within the growth cycle were examined by *in vivo* labelling of total cell protein with ^{35}S methionine. Cultures were initially characterised in fermenter based systems as this provided a homogeneous physical environment (Coppella and Dhurjati, 1989).

2.2.1 Changes in *de novo* protein expression patterns

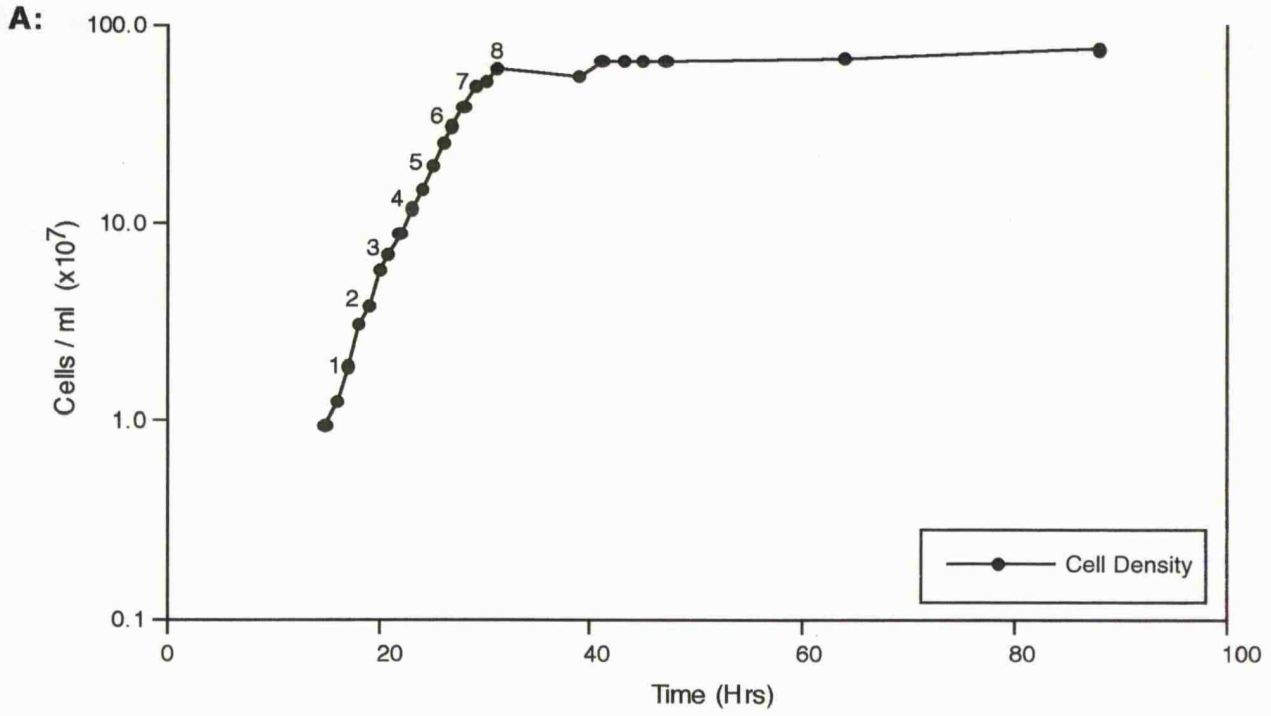
Saccharomyces cerevisiae strain S288Ca was grown overnight on blackstrap molasses, in a 1L LH series 500 fermenter, where the physical parameters; pH and %

DO2 were monitored, but not controlled and the culture temperature maintained at a constant 26°C (see Materials and Methods, section 9.3.4.1 for details of fermenter setup procedure). Cell samples were taken at intervals through respiro-fermentative and diauxic lag phases of growth after a cell density of 5.0x 10^6 cells ml^{-1} had been reached. These cell samples were used for ^{35}S methionine pulse-labelling of proteins and mRNA extraction for 'Northern' hybridization analysis (see Materials and Methods, sections 9.11 and 9.8.9, respectively). Cell density was determined with a haemocytometer and ethanol concentration was determined by enzymatic assay (see Materials and Methods, section 9.13.4).

De novo labelling showed changes in protein synthesis as cells moved from respiro-fermentative to diauxic lag (figure 2.1a & b). The appearance of certain proteins correlated with the predicted sizes of the cDNA gene products. In particular a protein at 14 kDa correlates with the expected size of the Hsp12p protein. The *HSP26* gene product could not be visualized by this technique as it does not contain methionine residues (Bossier *et al.*, 1989). Within the 30-35 kDa range, there is no detectable appearance of *de novo* proteins, which would correlate with the expression profiles for *ESP30* and *ESP35* (see below). A protein at ≈65 kDa showed a similar expression profile to that of the *ESP65* gene (see below).

2.2.2 Alteration in mRNA expression patterns

The observed pattern of *de novo* protein synthesis in yeast cells moving from respiro-fermentative to diauxic lag, indicated changes in gene expression occurred during this transition. Therefore, mRNA expression profiles for each of the isolated 'stationary phase' induced genes was examined and correlated to this pattern of protein synthesis. S288Ca cells cultured on blackstrap molasses showed a period of growth lasting for thirty-one hours (post-inoculation (p.i)), which reached a maximum cell density of 6.0x10^8 cells ml^{-1} (figure 2.2a). A progressive decrease in growth rate was observed over this period, possibly associated with the sequential assimilation of different carbon sources. Ethanol accumulated in the culture medium for twenty-three hours after inoculation, reaching a maximum level of 95 mmol L^{-1}. However, no apparent diauxic lag was observed and the transition to a respiratory phase of growth with the subsequent decrease in accumulated ethanol concentration, occurred at twenty-seven hours (p.i) (figure 2.2a). A specific growth rate of 0.10 hr^{-1}, with a decrease in % DO_2 after twenty-seven hours indicated that the cells were metabolizing ethanol by respiration, although there was no apparent increase in cell density (figure 2.2a). Yeast cells cultured on molasses have been reported to produce ethanol, even though they do not grow fully and eventually die (Feichter, Fuhrmann and Kappeli, 1981).



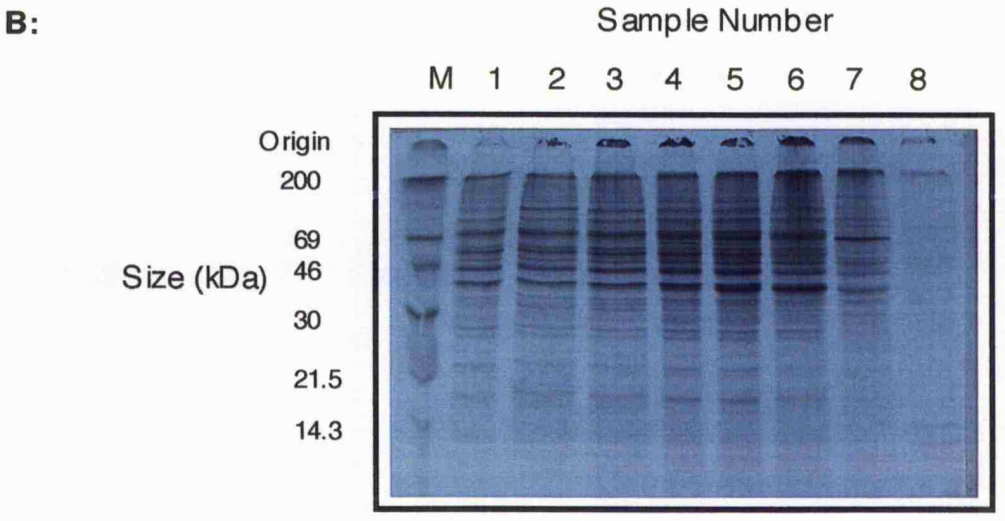


Figure 2.1 A and B: Protein synthesis during batch culture of S288Ca on blackstrap molasses. **A:** Growth curve of S288Ca on blackstrap molasses, sample points are shown by individual figures 1-8, for *in vivo* ^{35}S methionine labelling of proteins. Time indicated shows the number of hours after inoculation, with cell density indicated as log10x 10^7 cells ml^{-1}. **B:** Protein synthesis in molasses grown cells during the transition from exponential to stationary phase of growth between sample numbers 1-8 as shown in A. M indicates protein molecular weight marker Amersham [^{14}C] methylated protein rainbow® marker (MW 14,300-200,000). *In vivo* labelled proteins were resolved on a 15% polyacrylamide gel at 150 volts in a BioRad minigel apparatus for sixty minutes.

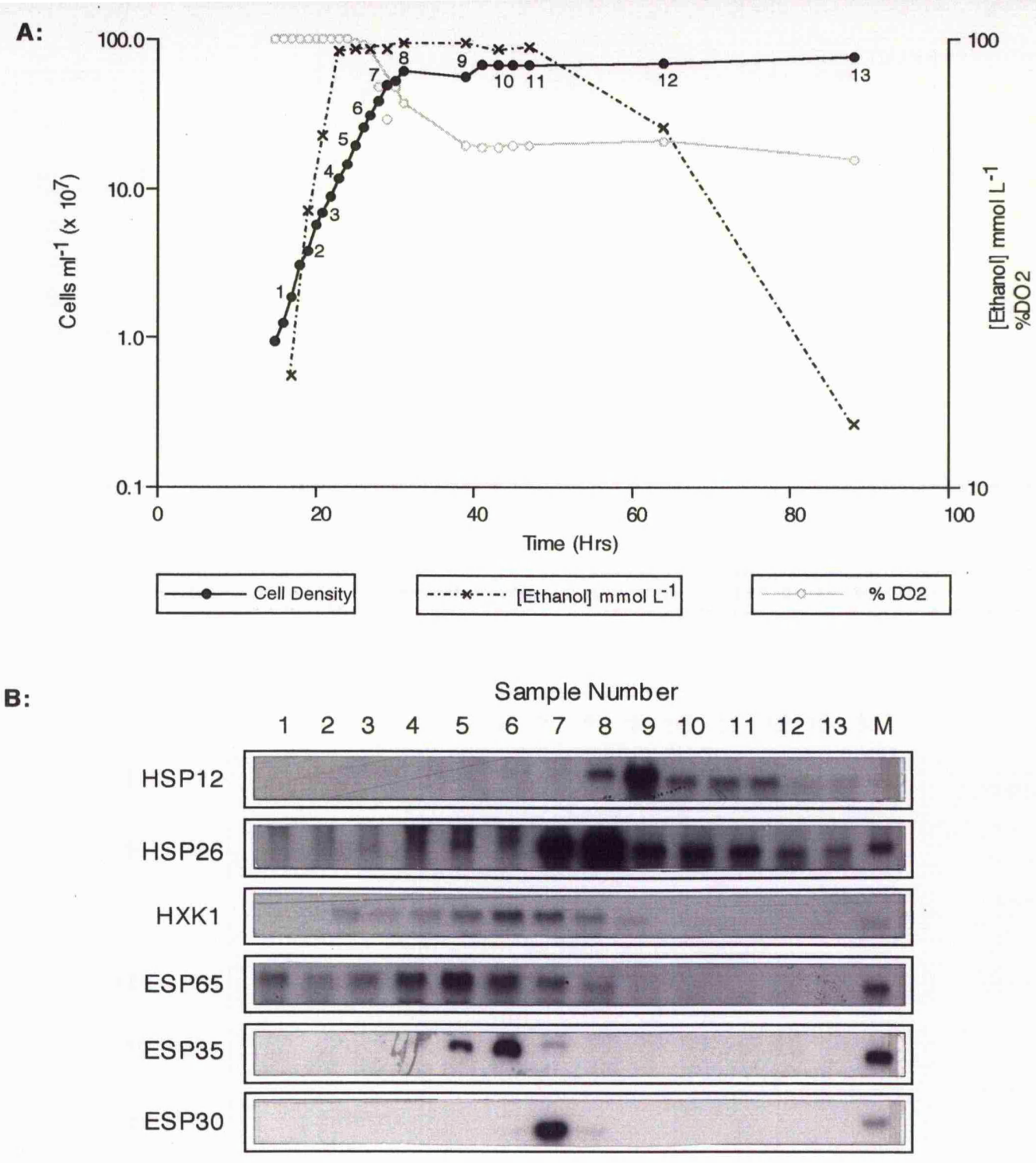


Figure 2.2 A and B: Gene expression pattern during batch culture of S288Ca on blackstrap molasses. **A:** Growth curve of S288Ca on blackstrap molasses indicating ethanol concentration (mmol L^{-1}) and sample points (shown by individual figures 1-13) for RNA analysis, *in-vivo* methionine labelling of proteins and ethanol concentration determination. Cell density is indicated as $\log^{10} \times 10^7$ cells ml^{-1}. **B:** 'Northern' hybridization of total cell RNA (10μg per lane) of batch culture grown at 26°C, extracted at set two hour time intervals after inoculation. Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. Numbers 1-13 indicate sample points shown in A. M is a total cell RNA sample known to shown expression of all stationary phase genes.

'Northern' hybridization analysis showed that *HSP12* induction occurred on entry into the respiratory/stationary phase of growth (at thirty-one hours (p.i)), whereas *HSP26* induction was prior to this at twenty-nine hours with maximal expression reached only on entry into stationary phase (figure 2.2b). *ESP65* was expressed during the respiro-fermentative phase of growth upto thirty-one hours (p.i), with cessation of expression on entry into the respiratory/stationary phase at thirty-one hours (p.i). *HXK1* expression was similar to that observed for *ESP65*; induction occurred in respiro-fermentative growth between twenty-one and thirty-nine hours (p.i). Maximum expression was reached within three hours of induction and then remained at a constant level upto thirty-one hours (p.i), where it then declined upon entry into stationary phase at thirty-nine hours (p.i).

ESP35 showed a transient period of expression, being induced at twenty-five hours (p.i) and rapidly declining, with no detectable hybridization signal as the cell culture entered into 'stationary' phase at thirty-two hours (p.i). *ESP30* showed a different pattern of expression to that of ESP35, with induction at thirty hours (p.i) and rapid decrease in hybridization signal intensity on entry into the 'stationary' phase (figure 2.2b).

2.3 Expression profiles in cultures grown on single carbon sources

As blackstrap molasses is a complex mixture, varying in nutrient and sugar composition, nutritional factors may affect gene expression and as induction of all the genes examined was associated with entry into diauxic lag/stationary phase, gene induction maybe due to the exhaustion of glucose and subsequent derepression. Therefore, examination of mRNA expression profiles in medium supplemented with individual carbon sources known to be present in blackstrap molasses might allow the identification of the control mechanisms acting upon these genes.

A number of carbon sources have been reported to be present in blackstrap molasses, including the catabolite repressing sugar (glucose), a non-catabolite repressing sugar (raffinose) and a non-fermentable carbon source (ethanol or lactate). The complex 'rich' medium (YEP), supplemented with a single carbon source, was used in the examination of induction profiles, in order to eliminate the possibility of any other nutrient becoming limiting. Expression profiles were examined by 'Northern' hybridization analysis in *Saccharomyces cerevisiae* strain S288Ca cells grown under homogeneous physical conditions in 1L and 16L LH series 500 and 2000 series fermenter systems. Cell density was monitored hourly and cell samples

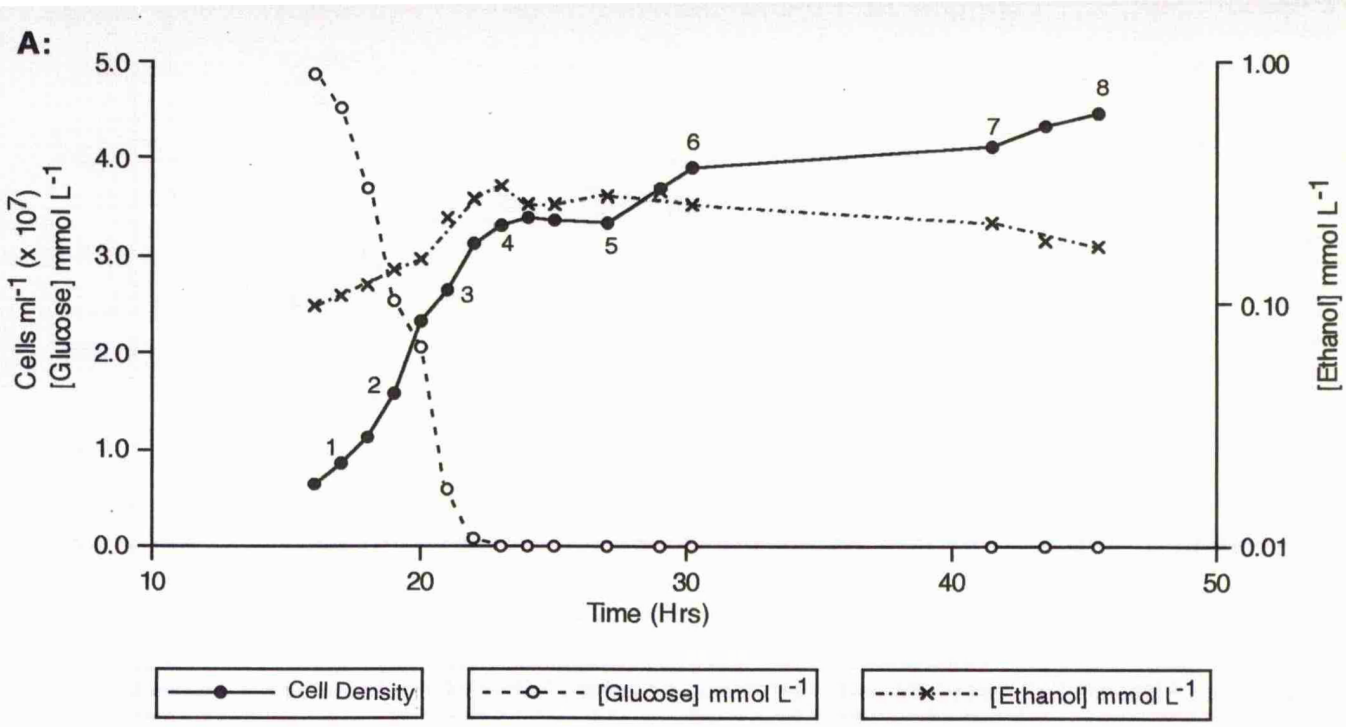
taken at intervals for mRNA isolation. Both supplemented carbon and ethanol concentrations were monitored throughout the culture growth using the appropriate enzyme assay systems (see Materials and Methods, section 9.13).

2.3.1 Glucose

Under aerobic conditions, glucose is the preferred substrate, in *S. cerevisiae*, where growth is governed by glucose regulation, leading to the characteristic diauxic pattern. In the first phase, formation of biomass is paralleled with glucose utilization and ethanol formation, while in the second growth phase ethanol serves as the substrate with derepression of respiration occurring on depletion of external glucose (Feichter *et al.*, 1981). Examination of gene expression patterns on YEP medium supplemented with glucose, would allow the identification of glucose repression effects.

Growth of S288Ca on glucose (figure 2.3a) showed a classic diauxic pattern associated with the utilization of glucose and suppression of respiration (Crabtree effect) followed by respiratory adaptation and a period of secondary growth involving oxidation of ethanol (Barford and Hall, 1979; Gadd, 1988; Toda, Yabe and Yamagata, 1980). The first period of exponential growth (upto twenty-one hours (p.i)), was characterized by a specific growth rate of 0.40 hr^{-1} and a corresponding decrease in glucose concentration, with accumulation of ethanol to a maximum level of 0.304mmol L^{-1}. On depletion of glucose (at twenty-two hours (p.i)), the yeast cell culture entered diauxic lag for approximately three to five hours, which was then followed by a second period of growth after twenty-seven hours (p.i), characterized by a specific growth rate of 0.02 hr^{-1} and an associated decrease in ethanol concentration (figure 2.3a).

Both *HSP12* and *HSP26* were induced upon entry into the diauxic lag at twenty-two hours (p.i), with expression continuing throughout the secondary growth phase (figure 2.3b). Induction of both genes corresponded with depletion in extracellular glucose, to a concentration of 0.96mmol L^{-1}, indicating a possible control by glucose repression. *HXK1* and *ESP65* (figure 2.3b) showed similar patterns of expression; induction occurred prior to entry into diauxic lag, with maximal expression at twenty-two hours (p.i). Expression then decreased to a basal level at the start of the secondary growth phase (twenty-seven hours (p.i)). *ESP35* showed weak induction at a late stage of ethanol respiration (after forty-one hours (p.i)) and *ESP30* was not expressed in this medium.



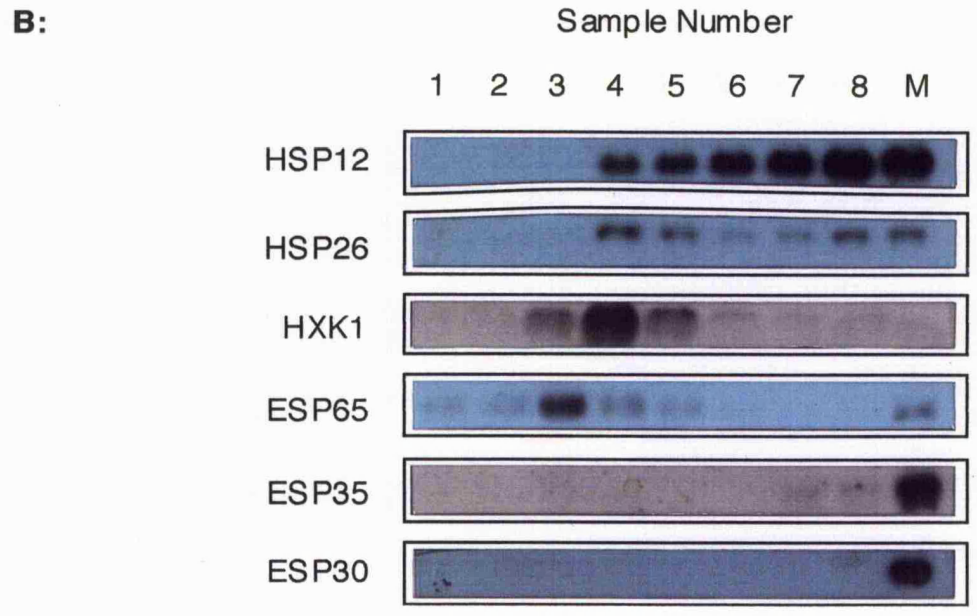


Figure 2.3 A and B: Gene expression pattern during batch culture of S288Ca on YEP medium supplemented with glucose. **A:** Growth curve of S288Ca on YEP+2% (w/v) glucose indicating glucose and ethanol concentration (mmol L^{-1}), sample points (shown by individual figures 1-8) for RNA analysis and carbon concentration determination. Cell density is indicated as $\log^{10} \times 10^7$ cells ml^{-1}. **B:** 'Northern' hybridization of total cell RNA (10μg per lane) of batch culture grown at 26°C, extracted at set two hour time intervals after inoculation. Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. Numbers 1-8 indicate sample points shown in A. M is a total cell RNA sample known to shown expression of all stationary phase genes.

2.3.2 Raffinose

Raffinose accumulates in molasses during the sugar refining process and is a trisaccharide of glucose, fructose and galactose (Barnett, 1981). Utilisation of raffinose in *Saccharomyces cerevisiae* is under the control of the *SUC* and *MEL* gene families (Carlson, 1987; Fraenkel, 1982) which specify invertase and α-galactosidase production, respectively. Invertase production is not induced by the substrates raffinose or sucrose (Rodriguez, Lampen and Mackay, 1981), but is subject to repression by external glucose (Stewart and Russell, 1987). Extracellular raffinose can be hydrolysed into fructose and melibiose by invertase (Barnett, 1981) and melibiose hydrolysed to glucose and galactose, by α-galactosidase. The non-repressing effect of raffinose is not fully understood, but it is thought, that as raffinose is only slowly hydrolysed by external invertase, the released fructose and glucose remain at non-repressing concentrations.

S288Ca grown on YEP+2% (w/v) raffinose showed a diauxic pattern (figure 2.4a); an initial period of exponential growth (μ=0.22 hr^{-1}) lasting twenty-five hours was followed by a slower growth phase after thirty hours (μ=0.02 hr^{-1}). External raffinose concentration fell to a basal level of 7.5mmol L^{-1} after twenty-five hours, accompanied by an increase in ethanol concentration to a maximum of 13.0mmol L^{-1} which corresponded with entry into the diauxic lag at twenty-one hours. A decrease in ethanol concentration was observed after twenty-three hours, but the secondary period of exponential growth (μ=0.02 hr^{-1}) only commenced after thirty hours (p.i) (figure 2.4a). The concentration of ethanol appeared to decline rapidly after the diauxic lag, whereas raffinose concentration appeared to remain at a constant level prior to entry into the diauxic lag. This apparently high basal level of raffinose maybe an artefact from the use of a combined enzyme assay. The assay used measures raffinose concentration by using galactose dehydrogenase to measure the amount of galactose released from raffinose by the action of α-galactosidase. Since the strain S288Ca is unable to utilise the galactose moiety released as it metabolises raffinose, accumulated galactose within the medium will contribute to the enzymatic determination, thereby giving an apparently measurable level even when all of the raffinose has been exhausted.

Under non-repressing conditions, both *HSP12* and *HSP26* (figure 2.4b) were expressed towards the end of the first period of exponential growth (twenty-three hours (p.i)). Both *HSP12* and *HSP26* induction were concomitant, however, HSP12 was expressed to a higher level (as indicated by hybridization signal intensity), but both showed a constant level of expression in ethanol respiring cells, after twenty-

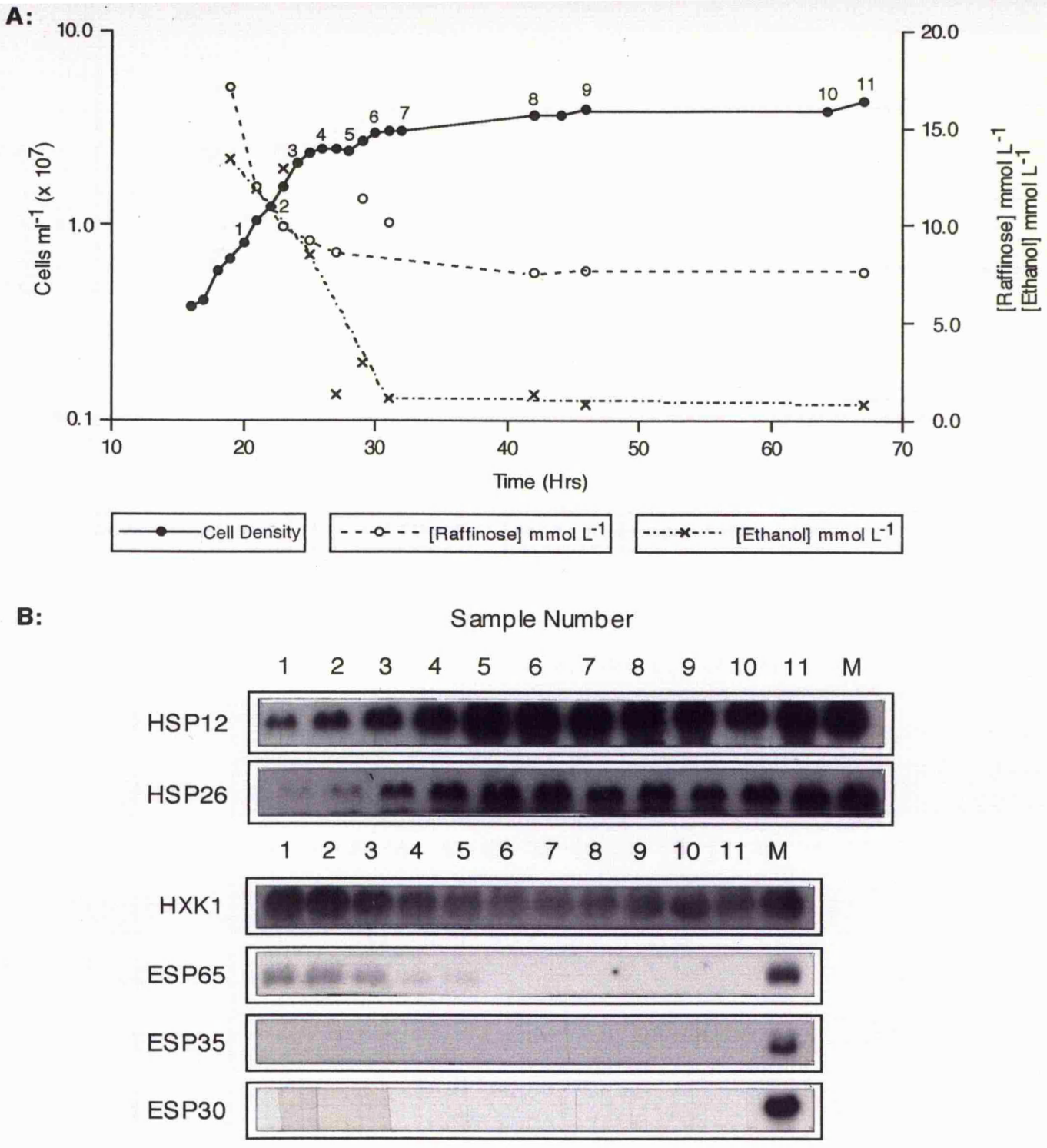


Figure 2.4 A and B: Gene expression pattern during batch culture of S288Ca on YEP medium supplemented with raffinose. **A:** Growth curve of S288Ca on YEP+2%(w/v) raffinose indicating raffinose and ethanol concentration (mmol L^{-1}), sample points (shown by individual figures 1-11) for RNA analysis and carbon concentration determination. Cell density is indicated as $\log^{10} \times 10^7$ cells ml^{-1}. **B:** 'Northern' hybridization of total cell RNA (10μg per lane) of batch culture grown at 26°C, extracted at set two hour time intervals after inoculation. Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. Numbers 1-11 indicate sample points shown in A. M is a total cell RNA sample known to shown expression of all stationary phase genes.

eight hours (p.i). *HXK1* was expressed throughout the early growth phase and then decreased upon entry into the diauxic lag, where expression levels then remained constant throughout the secondary growth phase. However, *ESP65* was only expressed during the first period of exponential growth (upto twenty-three hours (p.i)) after which a rapid decline was observed as the culture entered into diauxic lag. Both *ESP30* and *ESP35* were not expressed.

2.3.3 Lactate

S. cerevisiae is able to use a variety of non-fermentable carbon sources e.g. lactate, ethanol and glycerol. Lactate is assimilated into the cell by a proton-lactate symport (Leao and van Uden, 1986) and enters into the TCA cycle at the level of pyruvate (reviewed in (Fraenkel, 1982)). As only one molecule of ATP is generated per molecule of lactate, growth is very slow, with a low cell yield (Wills, 1990).

The growth pattern observed for yeast cells grown on YEP+2%(w/v) lactate showed a series of growth phases lasting over forty-three hours, after which cells entered stationary phase (figure 2.5a). The first distinct phase was upto nineteen hours (p.i), with a specific growth rate (μ) of 0.25 hr^{-1} and correlated with the accumulation of ethanol to 13.0mmol L^{-1}. A second period of growth, corresponding with a decrease in ethanol concentration was observed between nineteen and twenty-three hours (p.i). Growth rate decreased between twenty-three and thirty-seven hours (p.i), to reach a maxim density of 1.0x 10^8 cells ml^{-1}. This period of growth was accompanied by a decrease in ethanol concentration, to a basal level of 0.2mmol L^{-1} after thirty-seven hours, indicating growth was by oxidation of ethanol. A similar pattern was observed when cells were grown on glycerol (2%(w/v)) supplemented medium, except that the final biomass achieved on lactate was double that obtained on glycerol (data not shown).

HSP12 showed a constitutive pattern of expression during all growth phases (figure 2.5b), whereas *HSP26* showed a progressive increase in expression upto thirty-seven hours after inoculation and then declined as the yeast cells entered stationary phase. *HXK1* was not expressed on YEP+2%(w/v) lactate, however, *ESP65* did show a decline in expression level upto twenty-seven hours (p.i). *ESP35* showed a low level of induction at the point of entry into stationary phase, forty-one hours after inoculation. *ESP30* was not expressed.

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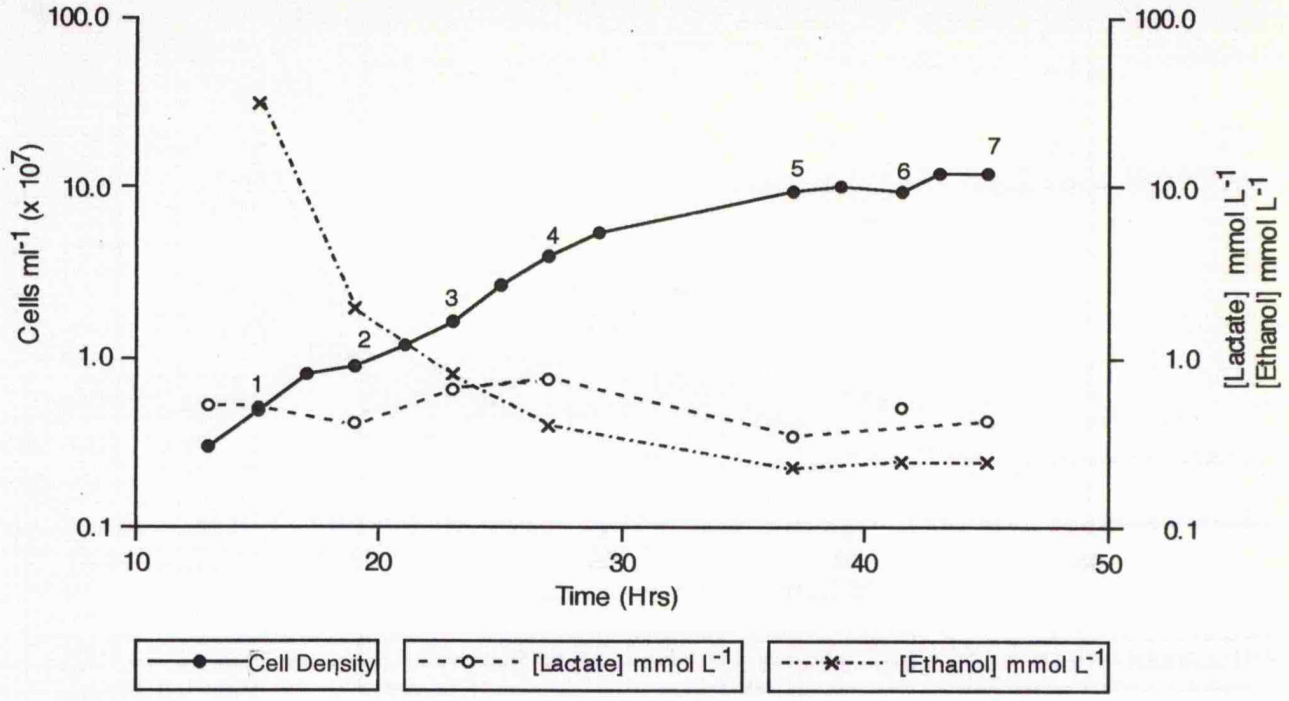

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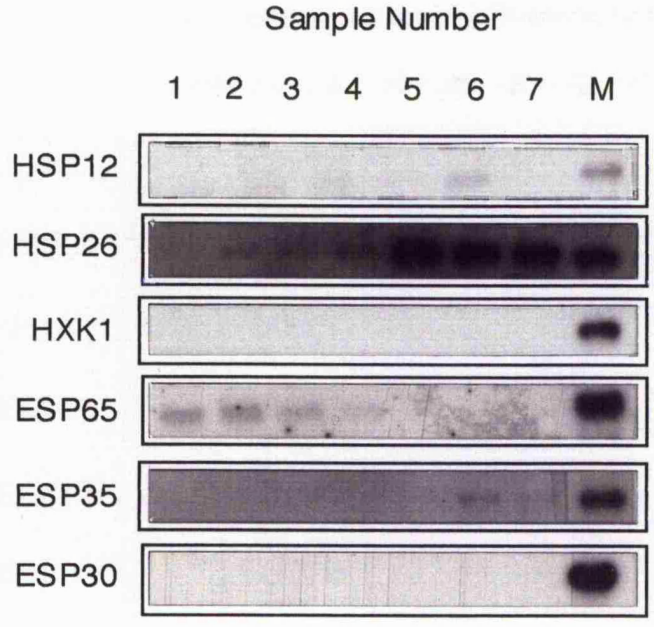


Figure 2.5 A and B: Gene expression pattern during batch culture of S288Ca on YEP medium supplemented with lactate. **A:** Growth curve of S288Ca on YEP+2% (w/v) lactate indicating lactate and ethanol concentration (mmol L^{-1}), sample points (shown by individual figures 1-7) for RNA analysis and carbon concentration determination. Cell density is indicated as $\log^{10}$ x10^7 cells ml^{-1}. **B:** 'Northern' hybridization of total cell RNA (10μg per lane) of batch culture grown at 26°C, extracted at set two hour time intervals after inoculation. Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. Numbers 1-7 indicate sample points shown in A. M is a total cell RNA sample known to shown expression of all stationary phase genes.

2.4 Expression summaries

Initial characterization of gene expression required the utilisation of fermenters in order to maintain a reproducible, homogenous physical environment. Preparation of the fermenter based systems employed in the initial characterization involved a complex procedure and since further studies required the culture of numerous mutant deficient strains (see chapter three), expression profiles were examined in cells cultured in shake flasks (see Materials and Methods, section 9.3.4.2 for shake flask setup details). Shake flask cultures have the advantage that they offer a relatively simple set-up procedure and are reported to produce similar conditions to those obtained within a fermenter system (Coppella and Dhurjati, 1989). To assess whether any difference in gene expression between fermenter grown cells and shake flask cultures did occur, expression profiles in cells grown in both systems were compared.

Growth of *S. cerevisiae* S288Ca was examined on a variety of individual carbon sources in complex media (YEP broth). Gene induction profiles were followed through various growth phases on repressing carbon sources (i.e. glucose, fructose and sucrose) and non-fermentable carbon sources (i.e. ethanol and glycerol). Yeast cells grown on either YEP+2%(w/v) glucose, 2%(w/v) fructose or 2%(w/v) sucrose showed the same diauxic growth and pattern of gene expression (data not shown), which was comparable with those obtained with fermenter grown cultures. Ethanol can be utilized as a primary carbon source by either respiration or gluconeogenesis, dependent upon the availability of oxygen (Haarasilta and Oura, 1975; Petrik, Kappeli and Fiechter, 1982) and the derepression of respiratory genes (Gadd, 1988). In ethanol and glycerol grown cells, a single exponential period of growth was observed, where *HSP12* and *HSP26* showed constitutive expression. *HXK1* appeared to be expressed on YEP+2% (v/v) ethanol at a low basal level, whereas *ESP65* was not expressed in either medium (data not shown).

2.5 Conclusions

The synthesis of particular cell proteins, identified by *de novo* pulse labelling, was correlated with the change in expression patterns of the set of 'stationary' phase genes. The expression patterns observed confirmed the profiles reported by (Praekelt and Meacock, 1990; Praekelt and Meacock, 1992). *HSP12* and *HSP26* showed a growth phase dependent expression pattern, being induced upon depletion of a repressing carbon source and expression during growth on non-fermentable carbon sources. This

suggests a possible glucose repression control. However, results obtained with glucose repression mutants and cAMP deficient mutants, suggest that *HSP12* and *HSP26* are not under a direct glucose repression control, but respond to a change in intracellular cAMP (see chapter three).

HXK1 induction was clearly subject to glucose repression, as expression was only observed on glucose exhaustion or during growth on non-repressing carbon sources. *ESP65* showed a similar pattern of expression. Therefore, it too maybe subject to glucose repression.

Expression of *ESP30* and *ESP35* was specific to cells grown on blackstrap molasses media and appeared not to be related to carbon source metabolism. *ESP35* expression occurred late into the secondary growth phase on YEP+2% (w/v) glucose, but was not observed in ethanol grown cells and therefore is not involved in ethanol catabolism. However, *ESP35* was expressed in cells cultured on SD medium + 2% (w/v) raffinose (a non-repressing carbon source) (Praekelt and Meacock, 1992), suggesting *ESP35* was repressed by a component(s) of YEP. Expression of *ESP30* and *ESP35* therefore appears not to be directly related to carbon metabolism.

Chapter 3:
Gene expression profiles in carbon catabolite repression and cAMP deficient mutants

3.1 Introduction

Expression profiles observed in glucose grown yeast cells showed that *ESP65* and *HXK1* expression was associated with the depletion of extra-cellular glucose, with both *HSP12* and *HSP26* induction correlated to entry into the diauxic lag and growth on non-fermentable carbon sources. *ESP30* and *ESP35* expression was shown to be unaffected by external carbon source and appeared to be specific to blackstrap molasses grown cells. As expression of these genes was associated with the depletion of glucose, a catabolite repression or a cAMP dependent control mechanism might be involved in their regulation. This was investigated by the analysis of expression profiles in the appropriate yeast mutants.

Two phenotypic classes of mutation have been identified in yeast which are involved in glucose repression; derepression mutants which are unable to derepress gene expression even when glucose is exhausted from the medium, e.g. *snf1* (*cat1, ccr1*) (Carlson *et al.*, 1981) and repression mutants which are resistant to glucose repression and show gene expression under repressing conditions, e.g. *tup1* (*flk1, umr1*) (Williams and Trumbly, 1990; Williams *et al.*, 1991) and *cyc8* (*ssn6*) (Carlson *et al.*, 1984; Trumbley, 1986). Another possible control pathway in the co-ordination of cellular response to external stimuli in yeast is via a cAMP dependent protein kinase (cAPK) (reviewed in (Broach, 1991; Matsumoto *et al.*, 1985)), where an increase in intra-cellular cAMP concentration activates a protein kinase (cAPK), which results with an increased phosphorylation of target proteins. When intra-cellular cAMP concentrations are low, the protein kinase is inactive, however, an increase in intra-cellular cAMP, results in the interaction of cAMP with the regulatory protein subunit, Bcy1p causing it to dissociate from the inactive holoenzyme complex and thereby release the active catalytic subunits. Therefore, in a *bcy1* mutant, the protein kinase is constitutively active and target proteins are phosphorylated. Mutation of the adenylate cyclase gene, *cyr1*, causes a reduction in the level of intra-cellular cAMP and results in the protein kinase remaining inactive, under all conditions. Hence genes under a cAPK control are constitutively expressed in a *cyr1* mutant. In the double mutant *bcy1/cyr1* the protein kinase remains active and shows a phenotype similar to that of the *bcy1* mutant.

3.2 Expression patterns in glucose repression mutants

In order to determine whether a glucose repression mechanism is responsible for the induction profiles observed, an isogenic series of glucose repression mutants, derived from S288C with deletions in *tup1*, *cyc8* and *snf1* genes (Williams and Trumbly, 1990; Williams *et al.*, 1991) were used in the examination of the expression profiles for *HXK1*, *HSP12*, *HSP26*, *ESP65*, *ESP35* and *ESP30* by 'Northern' hybridization. The phenotype of certain yeast mutants used in this study showed a high degree of cell flocculation and in order to maximise cell dispersal, baffled shake flasks were used to disperse these cell flocs (Stratford and Keenan, 1988).

Cultures were grown on YEP medium supplemented with 2% (w/v) glucose in 2L baffled shake flasks, at 30°C, 300 rpm. A yeast cell suspension sample (20ml) was taken every two hours after inoculation. Cells were collected by centrifugation and washed once in 250mM EDTA to completely disperse the remaining cell flocs (Stratford, 1989). After dispersal, cells were resuspended in an equal volume of sterile distilled water or EDTA solution and the cell density for each catabolite repression mutant was monitored by measurement of OD_{650} against an appropriate blank. Total cell RNA was prepared from the yeast cell suspension sample and the presence of the individual mRNA's monitored by 'Northern' hybridization to the appropriate cDNA clone (see Materials and Methods, sections 9.8.9 and 9.8.11).

The non-mutated parent strain, RTY235 ('wild-type') showed a normal diauxic growth pattern by fermentative metabolism of glucose (figure 3.1a), with entry into diauxic lag at twelve hours post inoculation, correlated with the exhaustion of extra-cellular glucose (figure 3.1a). The gene expression profiles observed for the six stationary phase genes were the same as those observed in S288Ca under the same conditions (see chapter two). Expression of both *HSP12* and *HSP26* was correlated with entry into a diauxic phase of growth and the depletion of glucose (at twelve hours post inoculation), with *HXK1* and *ESP65* induction corresponding to late exponential growth and entry into diauxic lag (upto twelve hours post inoculation). *ESP30* and *ESP35* were not expressed (figure 3.1b).

Genes under a glucose repression control cannot be derepressed even after glucose exhaustion in a *snf1* derepression mutant. Strain RTY333 (*cat1-42* (*snf1-42*)) showed no diauxie, with cells entering directly into stationary phase fifteen hours after inoculation and where complete exhaustion of glucose occurred after twenty four hours (post inoculation) (figure 3.2a). Both *HXK1* and *ESP65* showed a very reduced

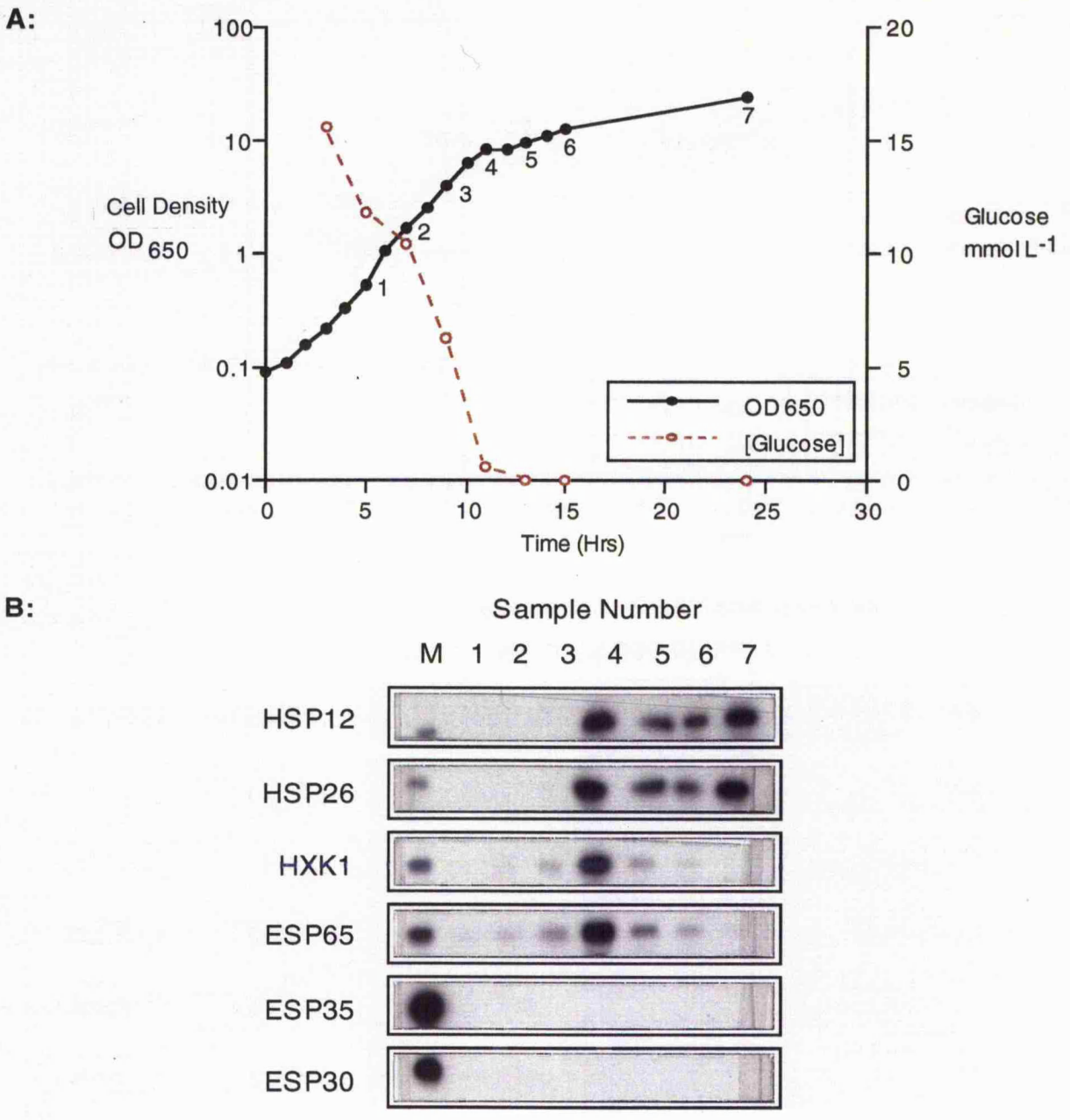


Figure 3.1 A and B: Gene expression profiles during batch culture of RTY235 (*Mata, his4-519, leu2-3,-112, trp1-289, ura3-52*) on YEP medium supplemented with glucose. **A:** Growth curve of RTY235 batch culture on YEP+2% (w/v) glucose, at 26°C. Cell density is indicated as adjusted optical density at 650 nm (OD_{650}) against a blank of 250mM EDTA solution. Sample points for RNA analysis are shown as individual figures 1-7. **B:** Northern hybridization of total cell RNA (10µg per lane), extracted at set two hour time intervals after inoculation (sample points 1-7 as indicated in A). Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. M is a total cell RNA sample known to shown expression of all stationary phase genes.

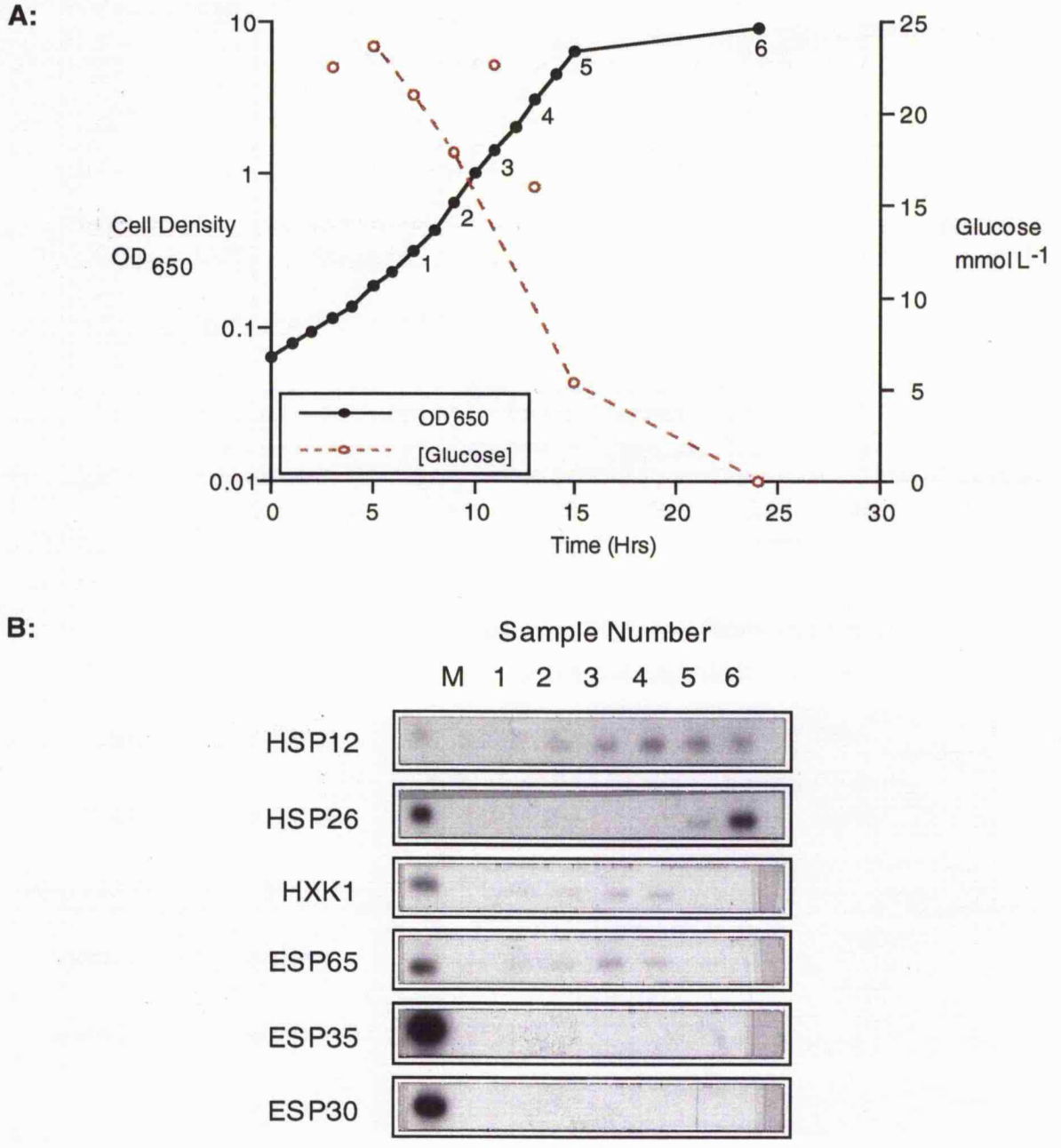


Figure 3.2 A and B: Gene expression profiles during batch culture of RTY333 (*Mata, leu2, trp1, ura3, cat1-42* (*snf1-42*)) on YEP medium supplemented with glucose. **A:** Growth curve of RTY333 batch culture on YEP+2% (w/v) glucose, at 26°C. Cell density is indicated as adjusted optical density at 650 nm (OD_{650}) against a blank of 250mM EDTA solution. Sample points for RNA analysis are shown as individual figures 1-7. **B:** Northern hybridization of total cell RNA (10µg per lane), extracted at set two hour time intervals after inoculation (sample points 1-7 as indicated in A). Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. M is a total cell RNA sample known to shown expression of all stationary phase genes.

constant basal level of expression, up until entry into stationary phase (twenty four hours post inoculation) (figure 3.2b). *HSP26* showed a similar pattern of expression to that observed in the non-mutated parent strain, RTY235 ('wild-type'). However, *HSP12* did show an earlier induction in the first period of exponential growth (at seven hours post inoculation), which corresponded to an extra-cellular glucose concentration of 21.0mmol L^{-1}. *ESP30* and *ESP35* were again not expressed.

In either a *cyc8* or *tup1* mutant, genes under a glucose repression are constitutively expressed. The double *cyc8*, *tup1* (RTY438) mutant has a similar phenotype of derepressed invertase expression, to that observed in the single *cyc8* and *tup1* mutants. However, RTY438 exhibits an increased invertase activity indicating that this mutant strain has a greater degree of glucose derepression than that observed in either of the single mutants, alone. When grown on YEP+2%(w/v) glucose, RTY438 (*tup1*-Δ1::TRPl+, *cyc8*-Δ1::LEU2+) cells exhibited a high degree of flocculation, with a less defined diauxic growth pattern (figure 3.3a). The entry into stationary phase correlated with the depletion of external glucose after twenty-four hours. Within the first exponential period of growth (repressing conditions) constitutive expression of both *HXK1* and *ESP65* was observed (figure 3.3b). *HSP26* showed some degree of derepression, where induction appeared to occur approximately four hours prior to entry into the secondary growth phase. *HSP12*, however, showed a similar expression profile to that observed in the 'wild-type'. Both *ESP30* and *ESP35* were not expressed.

As *tup1* or *cyc8* mutations suppress the effect of the *snf1* block on derepression, the expected pattern of expression in the double mutant *tup1*, *snf1* should be similar to that observed in the single *tup1* mutant (Williams and Trumbly, 1990). In the double mutant, RTY508 (*tup1*-Δ1::TRPl+, *snf1*-ΔlOO::URA3+), a diauxic pattern of growth was not observed (figure 3.4a), however, the yeast culture did exhibit a high degree of cell flocculation. Complete glucose utilization had occurred after fifteen hours (post-inoculation). The pattern of expression (figure 3.4b) was similar to that observed in the double derepression mutant RTY438 (*tup1*, *cyc8*), in that both *HXK1* and *ESP65* showed a constitutive expression pattern until entry into stationary phase after twenty four hours (post-inoculation). *HSP26* and *HSP12* showed a low basal level of expression in the first period of exponential growth, with an increase in expression level similar on entry into the secondary growth phase to that observed in the 'wild-type'. *ESP30* and *ESP35* were not expressed.

As expression of *ESP30* and *ESP35* appears to be specific to blackstrap molasses grown cells, the expression of these genes was examined in the mutants RTY235 ('wild-type'), RTY438 (*tup1, cyc8*) and RTY474 (*snf1*) grown on blackstrap molasses

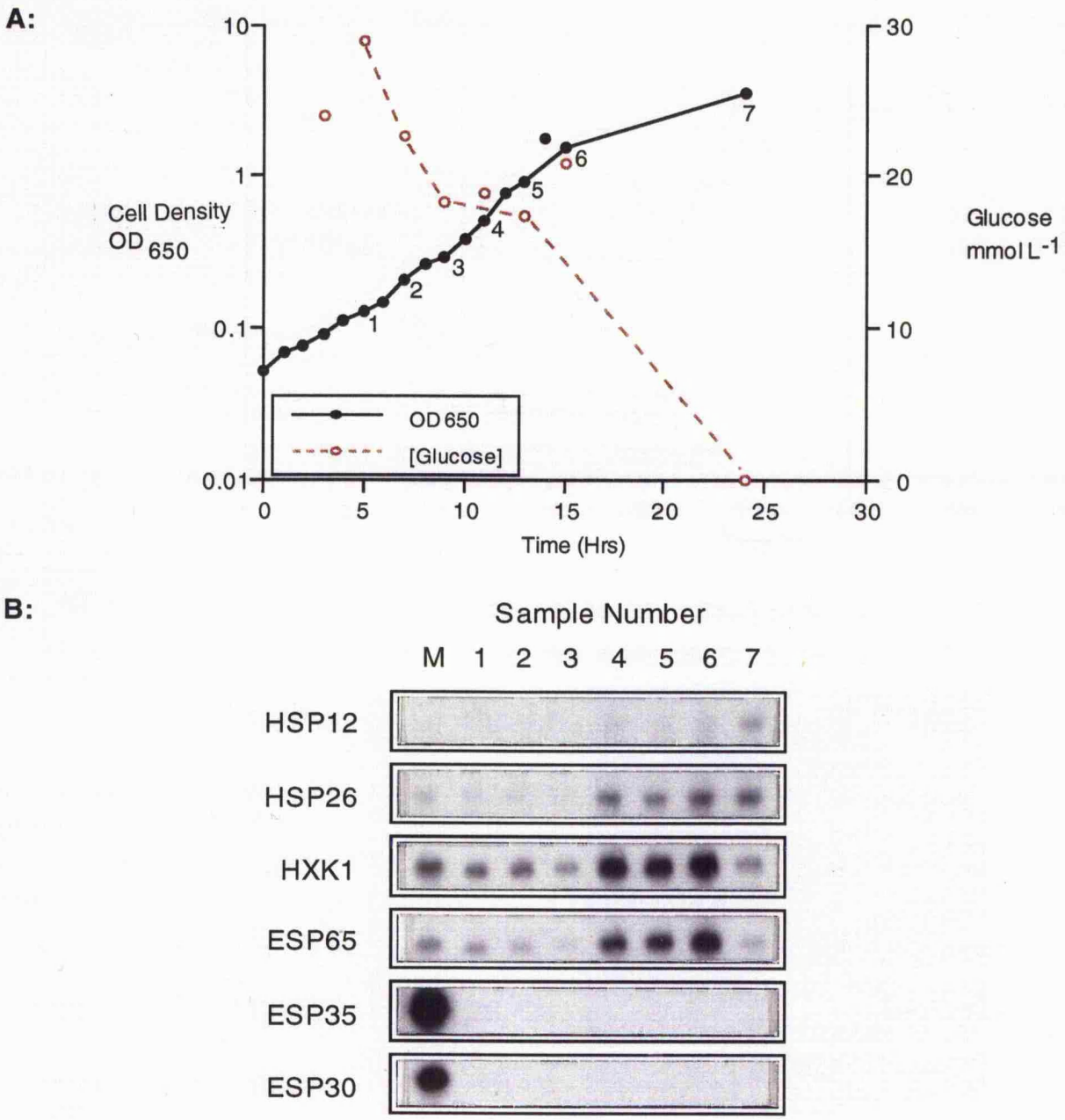


Figure 3.3 A and B: Gene expression profiles during batch culture of RTY438 (*Mata, his4-519, leu2-3,-112, trp1-289, ura3-52, tup1*-Δ1::TRP1+, *cyc8*-Δ1::LEU2+) on YEP medium supplemented with glucose. **A:** Growth curve of RTY438 batch culture on YEP+2% (w/v) glucose, at 26°C. Cell density is indicated as adjusted optical density at 650 nm (OD_{650}) against a blank of 250mM EDTA solution. Sample points for RNA analysis are shown as individual figures 1-7. **B:** Northern hybridization of total cell RNA (10μg per lane), extracted at set two hour time intervals after inoculation (sample points 1-7 as indicated in A). Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. M is a total cell RNA sample known to shown expression of all stationary phase genes.

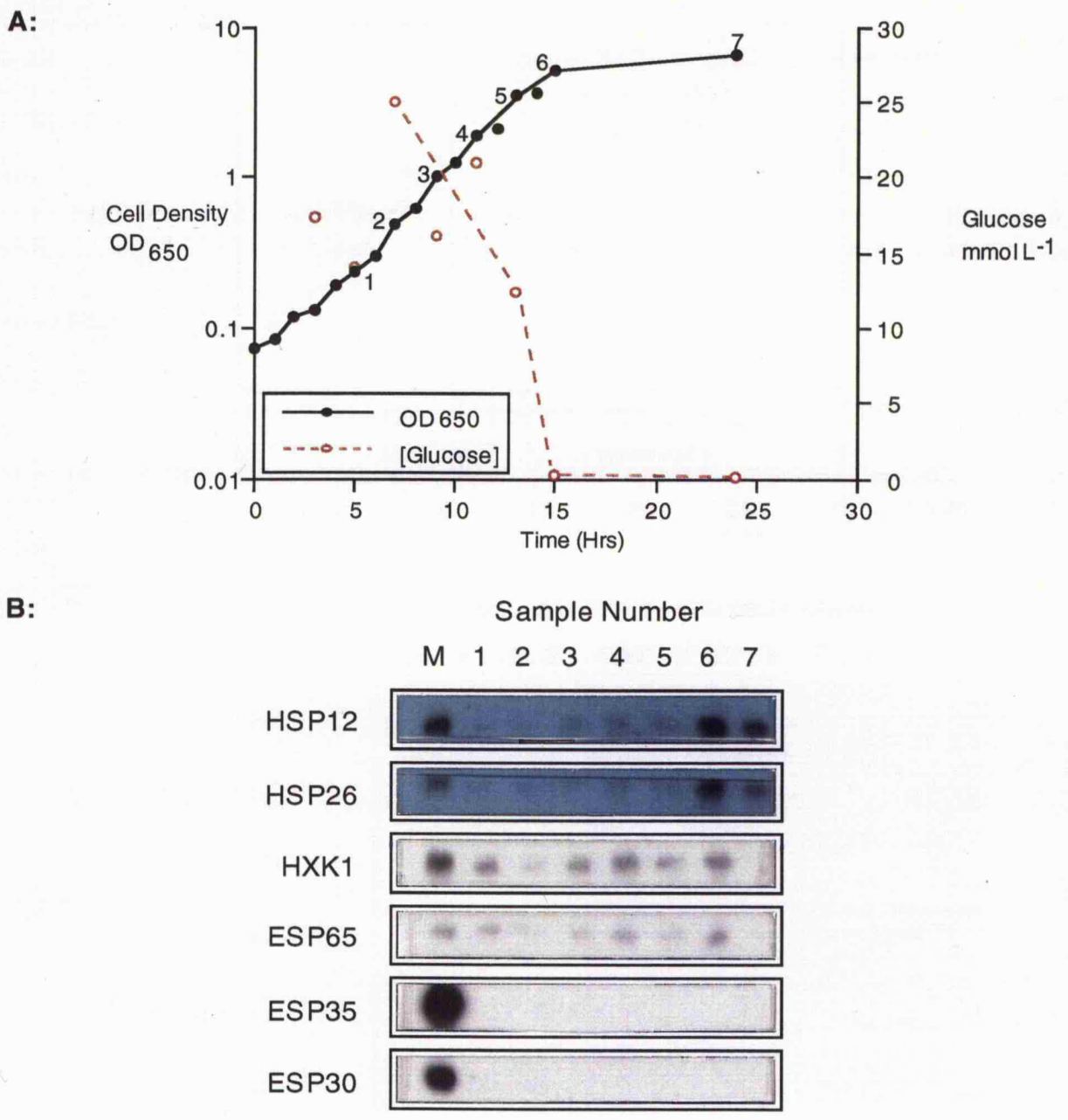


Figure 3.4 A and B: Gene expression profiles during batch culture of RTY508 (*tup1*-Δ1::TRP1+, *snf1*-Δ100::URA3+) on YEP medium supplemented with glucose. **A:** Growth curve of RTY508 batch culture on YEP+2% (w/v) glucose, at 26°C. Cell density is indicated as adjusted optical density at 650 nm (OD_{650}) against a blank of 250mM EDTA solution. Sample points for RNA analysis are shown as individual figures 1-7. **B:** Northern hybridization of total cell RNA (10µg per lane), extracted at set two hour time intervals after inoculation (sample points 1-7 as indicated in A). Hybridization probes were cDNA clones for *HSP12, HSP26, HXK1, ESP30, ESP35* and *ESP65*. M is a total cell RNA sample known to shown expression of all stationary phase genes.

medium. Starter cultures of these mutants in blackstrap molasses, supplemented with the appropriate amino acids, showed that growth of these mutants was significantly affected by the medium, in that both RTY235 and RTY438 showed a reduced doubling time and in the case of RTY474 (*snf1*), cell density hardly increased. 'Northern' hybridization analysis of shake flask cultures (data not shown) showed that expression of both *ESP30* and *ESP35* was unaffected and remained similar to that observed in 'wild-type' cells.

3.3 Expression patterns in cAMP deficient mutants

In order to ascertain whether a cAMP-dependent protein phosphorylation control mechanism effected expression of the six stationary phase genes, 'Northern' hybridization analysis was used to examine their induction profiles in a series of cAMP deficient mutants. Strains carrying single mutations of *bcy1*, *cyr1-2* and *bcy1/cyr1-2* (Matsumoto *et al.*, 1985; Smith, Dickinson and Wheals, 1990) were grown in YEP medium supplemented with 2%(w/v) glucose in shake flasks as previously described. Total cell RNA was extracted from yeast cells throughout the yeast growth phases. Cell samples representing exponential (Ex) and stationary phase (St), as determined by the stage within the growth phase, were used in the analysis of gene expression by 'Dot-blot' 'Northern' hybridization with the six stationary phase cDNA clones as probes (see Materials and Methods, section 9.8.10).

It is clear from the RNA 'dot-blot' analysis that both *HSP12* and *HSP26* are subject to regulation by the cAMP-dependent protein kinase. The stationary phase induction of both genes seen in 'wild-type' cells is prevented by the *bcy1* mutation which results in constitutive protein kinase (PK) activity; moreover the *cyr1-1* mutation, which reduces intracellular cAMP levels, causes constitutie expression of both genes (see figure 3.5). This regulation of *HSP12* and *HSP26* appears qualitatively similar but the 'dot-blot' analysis reveals quantitative differences. In the case of *HXK1* expression, there appears to be no significant effect of alterations in cAMP metabolism or cAMP-PK activity (see figure 3.5). *ESP65* shows an enhanced stationary phase expression in the *cyr1* mutant, which appears to be epistatic to the effects of the *bcy1* mutation; an explanation for this phenomenom is not immediately obvious, but may reflect interactions between the cAMP-system and the glucose-transport/catabolite repression pathway which does act on this gene.

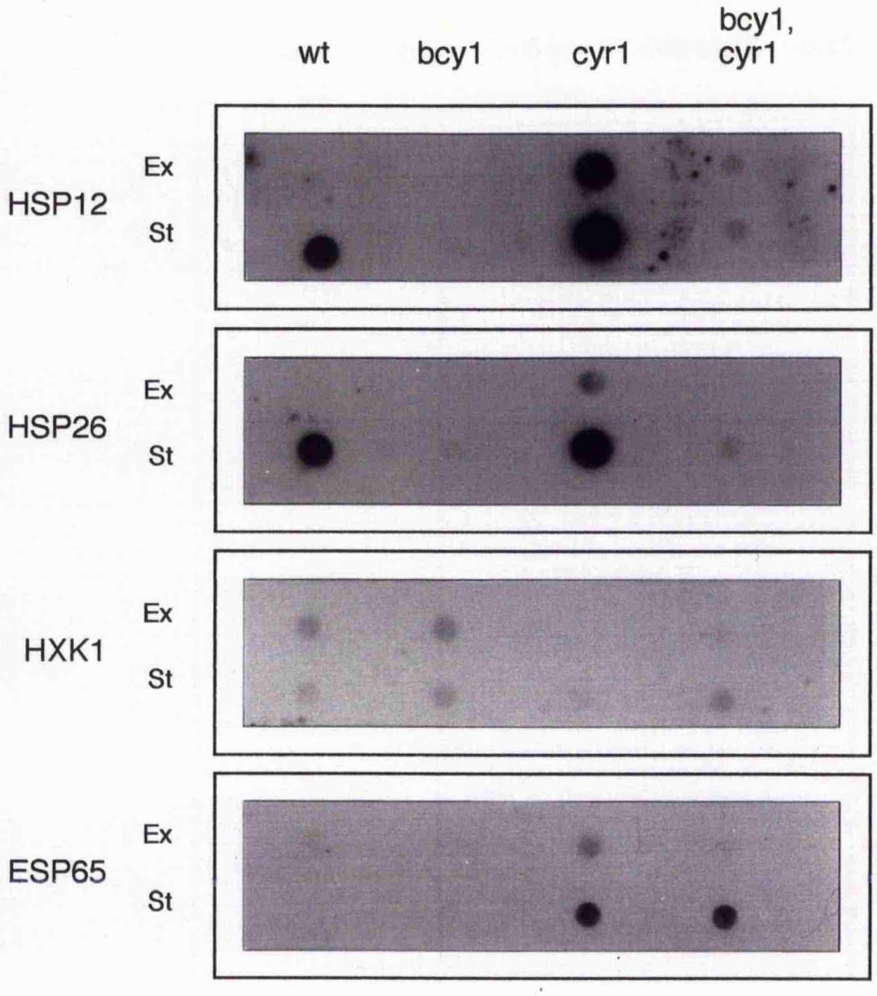


Figure 3.5: Expression of genes in mutants affected in cAMP dependent protein phosphorylation. Northern hybridization analysis of total cell RNA (5μg) to stationary phase cDNA probes. Cell cultures were grown in YEP medium supplemented with 2% (w/v) glucose and harvested in first period of exponential growth. Genotypes are 'wild-type' (wt), *bcy1* (bcy1), *cyr1-2* (cyr1) and *bcy1/cyr1-2* (bcy1,cyr1).

A summary of gene expression profiles in cAMP deficient mutants grown on YEP medium supplemented with 2% (w/v) glucose or on blackstrap molasses medium is shown in Table 3.1. Both *HSP12* and *ESP65* indicated a definite cAMP-dependent mechanism of control. *HSP26* appeared to show an incomplete response to intra-cellular cAMP levels, whereas *HXK1* expression remained unaffected. Expression profiles for both *ESP30* and *ESP35* in the cAMP deficient mutants grown on blackstrap molasses showed that they were both unaffected by intra-cellular cAMP concentration (data not shown).

3.4 Conclusions

HXK1 (hexokinase A) was previously identified and reported to be controlled via catabolite repression (Entian *et al.*, 1984; Kopetzki *et al.*, 1985). Within this study, *HXK1* has been shown to be regulated via a glucose repression control involving the *cyc8/tup1* pathway. The expression pattern observed in the *snf1* mutant confirmed that control of expression was via *SNF1*, as *CYC8* effectively by-passes the *snf1* block. Expression of *HXK1* was also found to be unaffected by cAMP levels.

ESP65 expression appears to be under a similar carbon catabolite control mechanism to that observed for *HXK1*. However, a cAMP dependent mode of regulation was also detected. As the pattern of expression was similar to that observed with *HXK1*, this could suggest that the *ESP65* gene product is involved in hexose/sugar metabolism.

HSP26 showed some degree of catabolite derepression and a possible cAMP dependent response. However, other repression mechanisms appear to be acting, as maximal expression levels were not observed in the catabolite mutants examined. The expression profiles observed seem to indicate that *HSP26* is induced in response to changes in the physiology of the cell detected by both catabolite derepression or a cAMP dependent protein kinase. As both of these mechanisms are detectors of the external cell environment, this mode of regulation could indicate that *HSP26* is possibly involved in cell survival, as the cell detects the change in external environmental conditions. 'Northern' hybridization data obtained differs from the expression profiles reported by (Silva *et al.*, 1994), where 'Western' hybridization data indicated that the Hsp26p was absent in a *bcy1* mutant and no detectable alteration in protein levels was observed in a cAMP deficient mutation.

TABLE 3.1: Summary of gene expression patterns observed in cAMP deficient mutants.

	GLUCOSE (YEP)												BLACKSTRAP MOLASSES			
	ESP65		HXK1		HSP12		HSP26		ESP35 (THI5)		ESP30 (THI4)		ESP35 (THI5)		ESP30 (THI4)	
	Ex.	St.	Ex.	St.	Ex.	St.	Ex.	St.	Ex.	St.	Ex.	St.	Ex.	St.	Ex.	St.
WT (S288Ca)	-	++	-	++	-	+++	-	++	-	-	-	-	-	+++	-	+++
BCY1 (AM180-2B)	-	+++	-	+	-	+	-	+	-	-	-	-	-	+++	-	+++
CYR1 (AM110-4C)	++	+++	-	-	+++	++++	++	+++	-	-	-	-	-	+++	-	+++
BCY1,CYR1 (AM9-10A)	-	++	-	++	-	-	-	+	-	-	-	-	-	+++	-	+++

KEY:

No expression is indicated by (-), expression levels are indicated by (+); intensity of the signal obtained by 'Northern' hybridization analysis with cDNA used as probe, is indicated by the number of (+), the larger the number, the higher the intensity observed. Growth phases are described as exponential growth (Ex.) and stationary phase (St.) as defined by Lewis *et al* (1993). Genotypes are as described in text (Matsumoto *et al* (1982,1985)).

Expression of *HSP12* is controlled via the cAMP triggered protein phosphorylation cascade (Praekelt and Meacock, 1990). Within a *snf1* mutant, *HSP12* expression was derepressed, which would suggest that *HSP12* is under a catabolite control. The expression pattern observed in the repression mutants, *tup1* or *cyc8* also support this observation. The mechanism by which *HSP12* is regulated could involve the phosphorylation sites involved in the cAPK mechanism of control. As *SNF1* and cAMP dependent protein kinase (cAPK) behave antagonistically (Thompson-Jaeger *et al.*, 1991) and affect some of the same cellular responses to nutrients (Hubbard *et al.*, 1992), the expression of *HSP12* would appear to be regulated via the same part of this pathway. The effect of cAMP on heat shock gene induction is not mediated through the heat shock element (HSE) (Piper, 1990) present in both the *HSP12* and *HSP26* promoters, therefore another element possibly the stress responsive element (STRE) may mediate the effect of cAMP on expression.

ESP30 and *ESP35* expression appears to be specific to yeast cells grown on blackstrap molasses medium. In both carbon catabolite and cAMP deficient mutants, no alteration in gene expression was detected, which would suggest that both of these genes are not under a carbon catabolite control mechanism and that induction appears to be repressed by a component of YEP; as carbon source has no effect on expression (see chapter two).

In conclusion, both *HXK1* and *ESP65* showed a catabolite repression control mechanism via *cyc8/tup1*. *ESP65* appeared also to be regulated by cAPK activity. Both *HSP12* and to a lesser extent *HSP26* showed a catabolite repression control, with *HSP12* also showing a distinct cAPK regulated activity. *ESP30* and *ESP35* both appeared not to be regulated via either by a cyclic AMP dependent kinase or catabolite repression mechanism.

Chapter 4:
ESP65 is the hexose transporter *LGT1*

4.1 Introduction

ESP65 cDNA was isolated by a differential screen of exponential and stationary phase mRNA from *S. cerevisiae* (Praekelt and Meacock, 1990). Expression profiles, under different nutritional conditions showed a distinct pattern of regulation, where *ESP65* was shown to be regulated via the *tup1*, *cyc8* pathway and also via a cAMP dependent protein phosphorylation mechanism. As the pattern of expression shown by *ESP65* was similar to that observed with the previously identified hexokinase P-I (*HXK1*) in *S. cerevisiae* (Entian *et al.*, 1984; Kopetzki *et al.*, 1985), which has been reported to be involved in glucose uptake and phosphorylation (Bisson and Fraenkel, 1983; Bisson and Fraenkel, 1984), this suggested that *ESP65* might also encode for a protein involved in carbon metabolism or transport.

4.2 *ESP65* is a member of a multigene family

Several multigene families are involved in carbon metabolism in yeast i.e. *MAL* (Needleman and Michaels, 1983), *SUC* (Carlson, Osmond and Botstein, 1980; Carlson *et al.*, 1981) and *HXK* (Bisson and Fraenkel, 1983) families. 'Southern' hybridization of restriction endonuclease digested genomic DNA, using the cDNA as a probe was used to determine whether *ESP65* was a unique gene or a member of a multigene family.

Genomic DNA (10µg) isolated from an overnight culture of S288Ca was digested with each of the following restriction endonucleases, individually; *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I. The restriction digestion mixtures were analysed by agarose gel electrophoresis and 'Southern' hybridization, using the *ESP65* cDNA clone as the probe (see Materials and Methods, sections 9.8.9 and 9.8.11). After a wash (3x SSC, 0.1%(w/v) SDS at 65°C), three hybridizing signals were observed in each restriction enzyme digest (figure 4.1). Within, the *Eco*RI restriction digest, four hybridizing regions are seen, indicating that the *ESP65* coding sequence may contain an internal *Eco*RI site. The *ESP65* cDNA therefore is homologous to three separate regions within the yeast genome, which would indicate that *ESP65* was a member of a multigene family. The size of each restriction fragment was not calculated, as only the copy number of the *ESP65* gene was of interest.

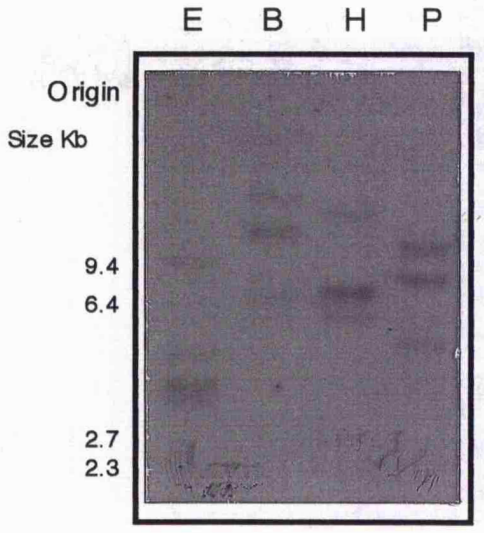


Figure 4.1: Copy number of the *ESP65* gene. Southern hybridization of restriction digest of *Saccharomyces cerevisiae* genomic DNA with the following restriction endonudeases *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). The size of fragments is not shown, however, the size marker (lambda *Hin*dIII) is illustrated. Southern hybridization was with the *ESP65* cDNA at 65°C in Church-Gilbert buffer, with washing at 65°C in 3xSSC, 0.1% SDS.

4.3 Partial sequence *ESP65* cDNA clone

ESP65 cDNA was obtained as a 1.1Kb *Eco*RI fragment and subcloned into the plasmid vector, pUC19 (Praekelt and Meacock, 1990). Partial DNA sequence determination of the cDNA clone, by dideoxy chain termination reactions with T7 DNA polymerase, was carried out in one direction only, using the universal M13/pUC (-47) forward sequencing primer.

Reliable DNA sequence was obtained over a length of two hundred and seventy nucleotides (nt) of the cDNA clone and was found to contain a continuous open reading frame of eighty-nine amino acid codons (figure 4.2). The DNA and inferred amino acid sequences were then compared to published DNA and polypeptide sequences held in the GenEMBL database. FASTA and TFASTA algorithms, which report only the 'best' region of similarity between two sequences based upon a 'word/dictionary' type search were used in the comparison of sequence data (Pearson, 1990; Pearson and Lipman, 1988). A FASTA search, showed a 100.0% identity over 269nt to the *S. cerevisiae* gene *LGT1* (*HXT4*), which corresponded to the region in *LGT1*, 672 to 941nt. Several other genes were also identified which showed a high percentage identity to the *ESP65* cDNA (Table 4.1). These included several members of the *S. cerevisiae HXT* multi-gene family.

A TFASTA search also found the amino acid open reading frame of *ESP65* cDNA (12 to 276nt), to be 100.0% identical, over 88 amino acid residues, to *S. cerevisiae LGT1*. The identified region corresponded to a proposed membrane spanning domain within *LGT1*, between amino acid residues 225aa to 313aa (Prior *et al.*, 1993). A number of other proteins, again all members of 'sugar transporter' families were also identified as showing a high percentage of identity towards the partial *ESP65* protein (Table 4.1). Thus, both FASTA and TFASTA searches identified the same genes, with the *ESP65* cDNA showing identity to a variety of hexose transport genes found in *S. cerevisiae*.

Alignment of primary amino acid sequence from members of the *HXT* family of hexose transport proteins showed a high degree of conservation in the primary amino acid sequence (figure 4.3). The region encoded by the *ESP65* cDNA is part of a membrane spanning motif which is highly conserved between all the identified hexose transport genes. Conservation of the primary structure between the different hexose transporter proteins indicates that these proteins have similar domains which are necessary for their function. Observed interdependence within the *HXT* family could be a result of this high degree of conservation between the different family members.

TABLE 4.1: Percentage similarity of *ESP65* cDNA sequence and inferred amino acid sequence. Genes identified by 'FASTA' and 'TFASTA' type search as showing identity against *ESP65* cDNA, *Saccharomyces* (Sc) and *Kluyveromyces* (Kl). Individual genes are identified as *LGT1* (Sc *RAG1*) (Prior *et al.*, 1993), *HXT3a* (*HXT4*) {Embl accession No. M81879}, *RAG1* (Goffrini *et al.*, 1990), *GAL2* (Nehlin, Carlberg and Ronne, 1989; Szkutnicka *et al.*, 1989), *HXT1* (Lewis and Bisson, 1991) and *HXT2* (Kruckeberg and Bisson, 1990).

GENE	Length	% identity
FASTA		
Sc LGT1 (Sc RAG1)	269 bp	100.0
Sc HXT3a (Sc HXT4)	269 bp	100.0
Sc HXT3	267 bp	75.3
Sc GAL2	269 bp	73.2
Sc HXT1	268 bp	70.9
Sc HXT2	269 bp	70.3
Kl RAG1	269 bp	70.3
TFASTA		
Sc LGT1 (Sc RAG1)	89 aa	100.0
Sc HXT3a (Sc HXT4)	89 aa	100.0
Sc GAL2	89 aa	75.3
Sc HXT3	89 aa	68.5
Sc HXT2	89 aa	67.4
Sc HXT1	89 aa	66.3
Kl RAG1	89 aa	66.3

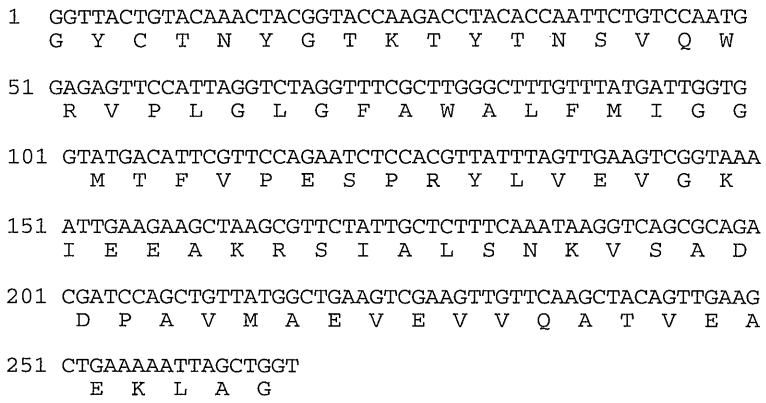
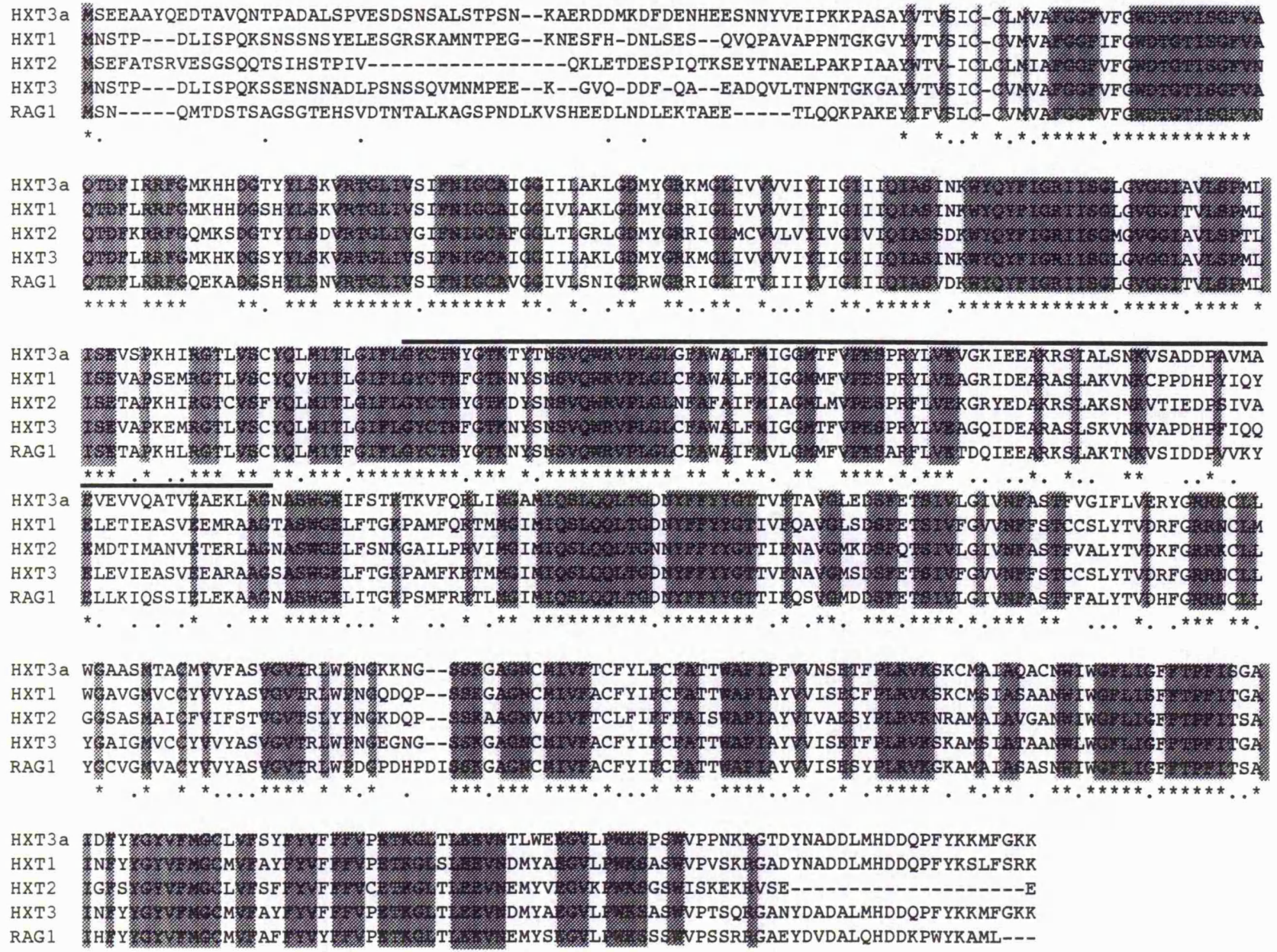


Figure 4.2: Partial DNA sequence *ESP65* cDNA. *ESP65* cDNA in pUC19 sequenced with the universal M13/pUC forward sequencing primer (-47) (New England Biolabs, NEB#1224), vector sequence is not illustrated and the deduced amino acid sequence is shown below the DNA sequence.

Figure 4.3: Alignment of hexose transporter primary amino acid sequences. Primary amino acid sequences were aligned by Clustal V (Higgins, Bleasby and Fuchs, 1992), with gaps introduced into the primary sequence to allow optimal alignment, indicated as '-'. Shaded areas ('*') indicate conserved regions between the individual polypeptide sequences and conserved functionally similar amino acids are indicated by '.'. The region of identity shown by the *ESP65* inferred polypeptide is indicated as a solid line. Designation of the individual proteins is as indicated in Table 4.1.


HXT3a
HXT1
HXT2
HXT3
RAG1

4.4 Conclusions

Comparison of sequence data showed that the *ESP65* cDNA is derived from the *S. cerevisiae* glucose transport gene, *LGT1* (Prior *et al.*, 1993). This gene has been described as a 'low' affinity glucose transporter, homologous to the glucose transporter *RAG1* in *K. lactis* (Goffrini *et al.*, 1989; Goffrini *et al.*, 1990). *LGT1* (*HXT4*) showed a high degree of amino acid conservation with *Kl. RAG1* and has been reported to complement the *rag-* phenotype (Prior *et al.*, 1993), which would suggest that these two genes are functionally similar.

Three separate hybridization regions were observed in the genome of *S. cerevisiae* to the *ESP65* (*LGT1*) cDNA clone, which suggest that *ESP65* (*LGT1*) is a member of a multigene family, unlike the *RAG1* gene in *K. lactis* which is present only as a single copy within the genome (Wesolowski-Louvel *et al.*, 1992). Multiple hybridization signals were reported when *S. cerevisiae* genomic DNA was hybridized to the *LGT1* gene, however, in the disrupted strain no detectable phenotype was observed (Prior *et al.*, 1993).

LGT1 has now been identified as *HXT4* (Prior *et al.*, 1993) and localised with *HXT1* onto chromosome VIII (Ko *et al.*, 1993). *HXT4* has been described as member of the hexose transport family, which includes *HXT1*, *HXT2*, *HXT3* and *SNF3*, but is not essential for cell viability (Ko *et al.*, 1993). Expression of any *HXT* gene is sufficient to allow growth on medium containing a high concentration of glucose, thus indicating the independence of function between these transporters (Reifenberger *et al.*, 1995).

Within *S. cerevisiae* there are two distinct hexose transport systems; 'high' affinity transporters and 'low' affinity transporters (Bisson, 1988). *HXT4* has been described as complementing the *snf3* phenotype (Ko *et al.*, 1993; Theodoris *et al.*, 1995). However, over expression of *HXT4* is reported to effect both 'high' and 'low' affinity uptake (Bisson *et al.*, 1993). As *LGT1* has been described as a 'low' affinity glucose transporter, disruption of which does not affect either 'low' or 'high' affinity uptake (Prior *et al.*, 1993) and as *LGT1* has been identified as *HXT4*, the affinity described for both genes must be questioned.

'High' affinity glucose transport genes in *S. cerevisiae* are controlled via a glucose repression mechanism (Bisson, 1988; Bustaria and Lagunas, 1986; Fuhrmann and Volker, 1992). Expression is reported to take place in cells entering stationary phase (Bisson and Fraenkel, 1984), where on depletion of the majority of the available hexose, a 'high' affinity transporter would be required for the efficient uptake of the

remaining hexose molecules. Recent reports have indicated that there is only one type of transport system, where transporters are constitutively expressed and their affinity for glucose changes when cells move from a repressed to a derepressed state (Walsh *et al.*, 1994; Wrede *et al.*, 1992). The proposed control of this change in the affinity of glucose transporter(s) is by binding of kinases (Walsh *et al.*, 1994), the presence of which has been correlated with glucose concentration, with kinases absent under 'low' affinity (i.e. high external hexose concentration) and present in 'high' affinity conditions (low external hexose concentration) (Bisson and Fraenkel, 1984).

ESP65 clearly showed a regulated pattern of expression via a carbon catabolite repression and a cAMP dependent phosphorylation mechanism, where expression was derepressed in *cyc8*, *tup1* mutants and repressed in a *snf1* mutant. This pattern of expression was the same as that reported for 'high' affinity glucose transporters, where *HXT2* (Wendell and Bisson, 1994) expression was derepressed in *ssn6* (*cyc8*) mutant and 'high' affinity transporters were repressed in *snf1* mutant (Bisson, 1988). *ESP65* also showed a cAMP dependent phosphorylation expression pattern, the same as that reported for 'high' affinity sugar transport genes, in which expression was absent or at low levels in a *bcy1* mutant and induced in *tpk1(w)* mutant (cAPK inactive) (Ramos and Cirillio, 1989; Riballo, Mazon and Lagunas, 1994).

Expression of 'high' affinity glucose transport proteins is associated with hexokinase expression (Bisson and Fraenkel, 1983), however, hexose phosphorylation is not involved in the transport mechanism (Nevado, Navarro and Heredia, 1994). Both *ESP65* and *HXK1* expression was observed to be correlated together dependent on growth phase and available carbon source, however, hexokinase P-I (*HXK1*) expression was not observed in cAMP deficient mutants. Although *ESP65* (*LGT1*, *HXT4*) has been described as a 'low' affinity transporter, which complements the 'low' affinity transporter *RAG1* in *K. lactis*, expression data suggests that *ESP65* encodes a 'high' affinity transport protein or has an inducible affinity phenotype. Regulation of the *HXT* family appears to be via three distinct regulatory mechanisms, induced by different levels of glucose. *HXT4* has recently been reported to be induced by low levels of glucose and repressed at high glucose concentration, where lack of induction at high glucose concentration is due to a glucose repression mechanism (Ozcan and Johnston, 1995).

From the FASTA comparisons (Table 4.1), it is evident that the cDNA clone is between 70-100% identical at the DNA level to other hexose transport genes identified in *S. cerevisiae*. It would appear from 'Northern' hybridization analysis that only a single transcript is detected, which would indicate that the other homologous

genes are either not transcribed under these conditions or that the hybridization of the cDNA probe did not occur to the homologous transcripts due to the high stringency conditions employed.

In conclusion, *ESP65* has been identified as the previously described 'low?' affinity glucose transport gene *LGT1* (=*HXT4*), a member of the multigene *HXT* family of *S. cerevisiae*. Expression was found to be regulated by a carbon catabolite and cAMP dependent protein phosphorylation mechanism, the same as that observed for 'high' affinity glucose transport proteins.

Chapter 5:
ESP30 cDNA is homologous to the thiamine regulated gene *nmt1a* of *Schizosaccharomyces pombe*

5.1 Introduction

Expression of both *ESP30* and *ESP35* was specific to yeast cells grown on blackstrap molasses medium, with induction profiles in S288Ca and isogenic catabolite repression mutants, which showed that neither gene was affected by carbon source or subject to carbon catabolite repression. However, *ESP35* was expressed after a prolonged period of secondary growth on YEP medium supplemented with 2% (w/v) glucose. As expression of both genes was associated with growth on blackstrap molasses, it suggested that a component(s) of YEP repressed expression of both genes.

ESP35 was subsequently reported to be repressed in yeast cells grown on blackstrap molasses medium supplemented with yeast extract, and was renamed *MOL1* (Praekelt and Meacock, 1992). The agent causing repression of *MOL1* was identified as thiamine. Consequently, *MOL1* was renamed and is presently termed, *THI4*. So, *ESP35=MOL1=THI4* and are different names for the same gene. *THI4* (*ESP35*) is now known to be involved in the biosynthetic pathway, leading to the formation of the thiazole precursor of thiamine (Praekelt *et al.*, 1994). As expression of *ESP30* was similar to that of *ESP35*, *ESP30* could therefore, have a similar function and control mechanism to that of *ESP35*.

5.2 *ESP30* expression is repressed by extracellular thiamine

ESP30 expression was similar to that observed for *ESP35* (*THI4*) when yeast cells were grown on blackstrap molasses medium (figure 2.2). As *ESP35* (*THI4*) had been shown to be repressed by an external thiamine concentration of 2.0μM (Praekelt *et al.*, 1994), an experiment was undertaken to assess whether *ESP30* expression was also regulated by extra-cellular thiamine. Induction profiles for both *ESP30* and *ESP35* in yeast cells grown on blackstrap molasses medium supplemented with thiamine-HCl at the repressing concentration of 2.0μM, were examined.

A S288Ca culture grown at 26°C on blackstrap molasses medium was used to inoculate two separate cultures; a control culture lacking extracellular thiamine and one culture supplemented with thiamine-HCl to a final concentration of 2.0μM.

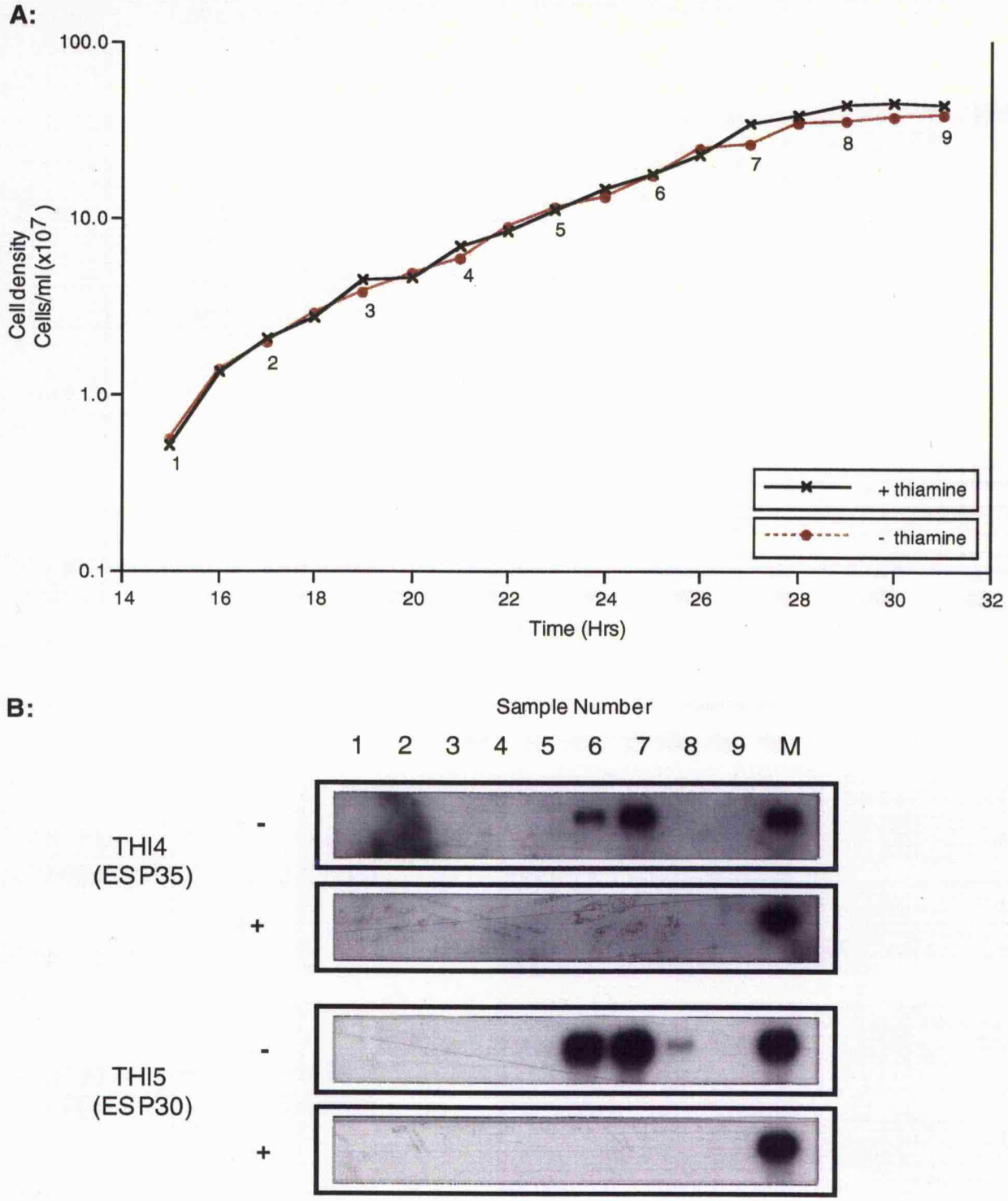


Figure 5.1 A and B: Growth curve of S288Ca on blackstrap molasses medium supplemented with +/- thiamine. **A:** Growth curve of S288Ca grown on blackstrap molasses and blackstrap molasses supplemented with 2.0μM thiamine HCl. Sample points are shown by individual figures (1-9) for RNA analysis. Cell density is indicated as cells ml^{-1} ($x10^7$). **B:** Northern hybridization of total cell RNA (5μg per lane) of batch cell culture grown on +/- thiamine supplemented blackstrap molasses medium, extracted at set two hour intervals after a cell density of 5.0 $x10^6$ cells ml^{-1} was reached. cDNA clones *ESP30* and *ESP35* (*THI4*) were used as hybridization probes. Numbers 1-9 indicate sample points in A. M is a total cell RNA sample known to show expression of *ESP30* and *ESP35* (*THI4*).

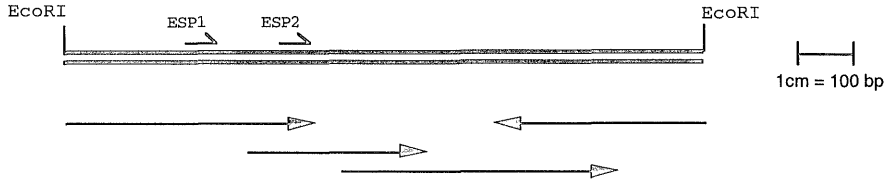
Cultures were inoculated to reach a cell density of 5.0x 10^6 cells ml^{-1} after fifteen hours of growth and were grown in parallel at 26°C, in shake flasks. Total RNA was extracted from twenty millilitre samples of cell suspension every two hours after a cell density of 5.0x 10^6 cells ml^{-1} was reached.

Growth of S288Ca in both supplemented and non-supplemented medium showed a similar growth pattern reaching approximately the same cell density after thirty-one hours (figure 5.1a). Expression profiles for *ESP30* and *ESP35* were examined in both cultures by 'Northern' hybridization analysis of total cell RNA, using the appropriate cDNA clone as the hybridization probe. No discernible alteration in the induction of both genes was observed (figure 5.1b), when cells were grown on non-supplemented medium, when compared to the previously described profile of yeast cells cultured on blackstrap molasses (see chapter two). However, in medium supplemented with thiamine-HCl (2.0μM), both *ESP30* and *ESP35* were completely repressed (figure 5.1b), suggesting that the *ESP30* gene was also regulated by extra-cellular thiamine in *S. cerevisiae*. This was confirmed by analysis of induction profiles on a chemically defined medium either supplemented or lacking thiamine-HCl (2.0μM) (see chapter seven).

5.3 Sequence of *ESP30* cDNA

Nucleotide sequence determination and comparison to known gene sequences was used to identify possible homologous genes to the *ESP30* cDNA. The *ESP30* cDNA was isolated as an approximate 0.6Kb fragment (Praekelt and Meacock, 1990) and cloned into an *Eco*RI site in the plasmid vector, pUC19. Universal sequence primers (M13/pUC reverse sequencing primer (-48) and M13/pUC sequencing primer (-47) (New England Biolabs)), priming from the flanking regions within the vector DNA were used in the initial sequence reactions. Oligonucleotide primer-walking was then used to bridge the region between 250 to 449nt which were not covered by the universal primers. Primers were chosen to hybridize approximately fifty nucleotides before the limit obtained from the previous sequencing reactions and were designed with an approximate 50% GC content (where possible) (Strauss *et al.*, 1986).

The nucleotide sequence of the *ESP30* cDNA clone was 558 nucleotides (nt) in length (excluding added cloning sites) (figure 5.2) and contained a continuous open reading frame of 140 codons. As the DNA sequence represents a cDNA, downstream of the identified open reading frame are several characteristics associated with the 3' region of a transcribed yeast gene, with the polyadenylation tail, between nucleotides




```
1    GAATTCCTTTACATCTGCAACGATGAATTTTTGAAGAAGAACCCTGAAAA
           L  Y  I  C  N  D  E  F  L  K  K  N  P  E  K

51   GGTCAGAAAGTTCTTGAAAGCCATCAAGAAGGCAACCGACTACGTTCTAG
      V  R  K  F  L  K  A  I  K  K  A  T  D  Y  V  L  A
     _______ESP1_______
101  CCGACCCTGTGAAGGCTTGGAAAGAATACATCGACTTCAAGCCTCAATTG
       D  P  V  K  A  W  K  E  Y  I  D  F  K  P  Q  L

151  AACAACGATCTATCCTACAAGCAATACCAAAGATGTTACGCTTACTTCTC
     N  N  D  L  S  Y  K  Q  Y  Q  R  C  Y  A  Y  F  S
                                 _________ESP2_______
201  TTCATCTTTGTACAATGTTCACCGTGACTGGAAGAAGGTTACCGGTTACG
      S  S  L  Y  N  V  H  R  D  W  K  K  V  T  G  Y  G

251  GTAAGAGATTAGCCATCTTGCCACCAGACTATGTCTCGAACTACACTAAT
       K  R  L  A  I  L  P  P  D  Y  V  S  N  Y  T  N

301  GAATACTTGTCCTGGCCAGAACCAGAAGAGGTTTCTGATCCTTTGGAAGC
     E  Y  L  S  W  P  E  P  E  E  V  S  D  P  L  E  A

351  TCAAAGATTGATGGCTATTCATCAAGAAAAATGCAGACAGGAAGGTACTT
      Q  R  L  M  A  I  H  Q  E  K  C  R  Q  E  G  T  F

401  TCAAGAGATTGGCTCTTCCAGCTTAAACGCACTTCGTCACTCCTCGAGCG
       K  R  L  A  L  P  A  *

451  CAGCCTTCCTCTACGACTTGTGTATGATTTCACATTAGGTTTTTTTTCCT

501  CCCTGATATTTAGATATTTACTATTAAGATATTATTTATGAGCCTTTTAT

551  TAAAAAAAAAACCGAATTC
```

Figure 5.2: DNA Sequence of *ESP30* cDNA. The *Eco*RI cloning sites are underlined within the DNA sequence shown. Amino acids of the inferred open reading frame are shown under the DNA sequence. The DNA sequence representing the 3' untranslated region (containing the polyadenylation tail) is shown by outline of the DNA sequence. Oligonucleotide primers used in sequence analysis are shown by overline, with the designation of the primer (Appendix I). The orientation of the primers is indicated on the line diagram. Arrows indicate the length and orientation of the DNA sequence.

426 to 563nt and the TAA termination codon at position 424nt. Comparison of this inferred amino acid sequence using TFASTA analysis (Pearson, 1990; Pearson and Lipman, 1988) to the GenEMBL sequence database, identified the *ESP30* polypeptide (1 to 139aa) as 46.9% identical to the thiamine regulated gene *nmt1a* of *S. pombe* (figure 5.3).

5.4 *ESP30* is a member of a multigene family

The thiamine regulated gene *ESP35* (*THI4*) was detected as a single copy in the *S. cerevisiae* genome, after high stringency hybridization with the respective cDNA clone. However, at a lower stringency other hybridizing regions were detected indicating other related sequences are present within the yeast genome (Praekelt and Meacock, 1992). *ESP30* copy number was examined in the same way, by hybridization of the cDNA clone to genomic DNA digested with a number of restriction endonucleases.

Genomic DNA (10µg), extracted from an overnight culture of S288Ca grown on YEP medium supplemented with 2%(w/v) glucose was digested with the individual restriction endonucleases; *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I. The restriction digests were analysed by agarose gel electrophoresis and 'Southern' hybridization, using the *ESP30* cDNA clone as probe. After a high stringency wash (see Materials and Methods, section 9.8.8), the *ESP30* cDNA probe was found to hybridize to four separate regions within the genomic DNA, in each of the different restriction endonuclease digests (figure 5.4). Within the *Hin*dIII digest, three hybridization regions are observed, however, one hybridization signal is double the intensity of the other signals and indicates on a shorter autoradiographic exposure to be comprised of two hybridization regions (data not shown). Since none of these enzymes cuts within the cDNA, the multiple signals detected must represent multiple copies of the *ESP30* sequence within the yeast genome. *Eco*RI generated genomic DNA fragments of sizes 2.8, 3.2, 4.0 and 9.3Kb were detected (hybridization regions are marked in figure 5.4 by black triangles), which corresponded to the size of *Eco*RI fragments isolated in λ clones isolated from a genomic library (see chapter six).

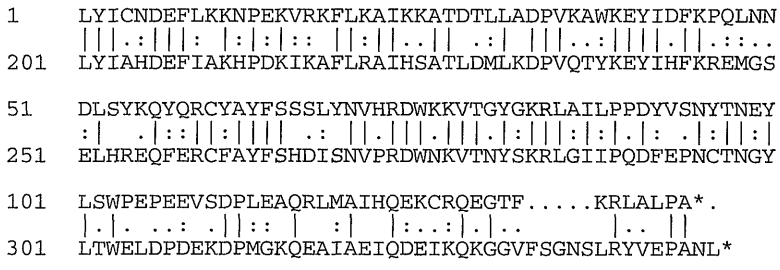


Figure 5.3: TFASTA comparison of the *ESP30* cDNA. The inferred open reading frame of 140 amino acid residues between nucleotide positions 11 to 430nt of the *ESP30* cDNA, showed a 46.9% identity with *Schizosaccharomyces pombe nmt1a* peptide. The *ESP30* cDNA inferred peptide sequence (top), *Sc pombe nmt1* sequence (bottom), where ' | ' is an exact match between residues, ':' is a match between similar amino acids and ' . ' is a neutral substitution.

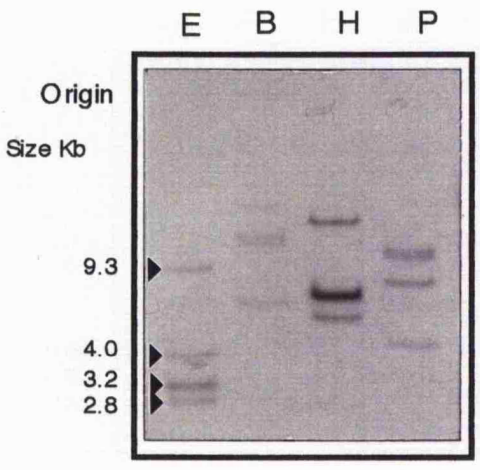


Figure 5.4: Copy number determination of *ESP30* gene in *Saccharomyces cerevisiae* genome. 'Southern' hybridization of S288C a genomic DNA digested with the following restriction endonudeases; *Bam*HI, B; *Eco*RI, E; *Hind*III, H; *Pst*I, P. Hybridization was with the *ESP30* cDNA done in Church-Gilbert buffer at 65°C, followed by two high stringency washes (0.1% (w/v) SDS, 3x SSC) at 65°C.

5.5 Conclusions

ESP30 and *ESP35* (*THI4*) expression was only observed during growth of yeast cells on blackstrap molasses medium. *ESP35* (*THI4*) expression was confirmed to be repressed by extracellular thiamine (Praekelt *et al.*, 1994), with a similar repression of *ESP30* observed.

Comparison of the DNA sequence and inferred peptide sequence of the *ESP30* cDNA clone indicated homology to the thiamine regulated gene *nmt1* of *S. pombe,* (Maundrell, 1990), which encodes a gene product involved in the synthesis of the pyrimidine moiety of thiamine (Schweingruber *et al.*, 1991). *ESP30* appeared to be a member of a multigene family in *S. cerevisiae* consisting of four members, whereas the *nmt1* gene is reported to be present as a single copy within the *S. pombe* genome (Maundrell, 1990).

Recently another gene, *nmt2*, has been isolated in *S. pombe*, and is reported to be co-ordinately regulated with *nmt1a* (Manetti *et al.*, 1994). Both *ESP30* and *ESP35* (*THI4*) appear also to show a coordinate form of regulation by thiamine, similar to that reported for *nmt1a* and *nmt2* in *S. pombe*. The *nmt2* gene product showed a 66.6% identity over 219 amino acid residues to the *S. cerevisiae ESP35* (*THI4*) peptide, which is involved in the synthesis of the thiazole moiety of thiamine (Praekelt *et al.*, 1994); the same function has been ascribed to *nmt2* (Manetti *et al.*, 1994; Zurlinden and Schweingruber, 1992).

Chapter 6:
Isolation and analysis of individual gene members of the *ESP30* family

6.1 Introduction

In order to characterize members of the *ESP30* family, individual family members needed to be isolated. This can be achieved by the use of a genomic library constructed in a suitable vector followed by hybridization screening with the radiolabelled cDNA clone as a probe. A genomic library comprises a collection of bacteriophage, plasmid or cosmid clones containing randomly generated DNA fragments covering the entire genome, where the number of clones needed to constitute a fully representative library is dependent upon the size of the genome and the size of the fragments cloned. To ensure the production of a representative library, the minimum library content should be equivalent to ten genome's worth of randomly generated DNA fragments. As the *S. cerevisiae* haploid genome is 1.5×10^7 bases in size, the most convenient library is one constructed in a bacteriophage replacement vector. This system has a high cloning efficiency (accepting an insert DNA 15-22Kb in size), a simple screening procedure and would cover the entire genome of *S. cerevisiae* in 10^4 clones.

6.2 Construction of a yeast genomic DNA library in λ EMBL3

A genomic DNA library, was constructed from *Saccharomyces cerevisiae* (S288Ca) genomic DNA in the bacteriophage replacement vector, λ EMBL3 (Fischauf *et al.*, 1983). The protocols of (Hadfield, 1987) and (Ausubel *et al.*, 1989) were used throughout the construction and screening of this library. Two polylinker sequences in λ EMBL3 facilitate the cloning of DNA fragments generated by the restriction endonucleases; *Bam*HI, *Eco*RI and *Sal*I or any other endonucleases that produce compatible cohesive termini. This design of the polylinker sequences simplifies the preparation of the vector for cloning, where DNA fragments of 15-20Kb in size generated by partial digestion with *Sau*3A can be ligated into the vector *Bam*HI restriction sites. The restriction endonuclease, *Sau*3A recognizes the 4-base sequence; 5' |GATC 3' and was chosen to produce the partial digest of genomic DNA as this endonuclease would generate a more random collection of insert fragments than endonucleases that recognize 6-base sequences.

Genomic DNA was isolated from sphaeroplasts of 'wild-type' *S. cerevisiae* strain, S288Ca (see Materials and Methods, section 9.5.2.1). Conditions for the *Sau*3A partial digestion of genomic DNA, which would generate 15-20Kb DNA fragments was initially determined by small scale digestion, by varying reaction times and restriction endonuclease concentration (see Material and Methods, section 9.8.1; see also (Maniatis, Fritsch and Sambrook, 1982)). Analysis of the reaction mix by agarose gel electrophoresis identified the optimal conditions as 0.01u *Sau*3A per µg genomic DNA, incubated at 37°C for two minutes. Based upon these conditions, 400µg genomic DNA was digested with 0.01u *Sau*3A per µg DNA and the degree of digestion determined by agarose gel electrophoresis of a 10µg sample. Fragmented genomic DNA was extracted once with phenol/$CHCl_3$/IAA (see Materials and Methods, section 9.4), ethanol precipitated and resuspended in a final volume of 400µl sterile distilled water (see Materials and Methods, section 9.8.5). *Sau*3A generated DNA fragments were resolved on a continuous 10% to 40% (w/v) sucrose density gradient (see Materials and Methods, section 9.5.3). The sucrose density gradient was fractionated and each fraction analysed by gel electrophoresis to determine the fraction(s) containing 15Kb sized DNA fragments (figure 6.1). Fraction numbers 3 to 9 (figure 6.1) contained DNA fragments sized to approximately 15Kb; the most appropriate sample was fraction number 5 which was used in the subsequent steps for library construction.

Commercial bacteriophage λ EMBL3 *Bam*HI vector arms (Promega) were ligated at 25°C for three hours, to 15Kb sucrose gradient purified, genomic DNA fragments at varying concentrations of insert DNA to vector DNA (see Materials and Methods, section 9.8.4). The ligated products were packaged as outlined into bacteriophage A particles using a commercial packaging extract (Promega Packagene®) (Promega, 1991). The packaged library was then stored at 4°C with $CHCl_3$, prior to transfection into the *E. coli* host strain, LE392 (see Materials and Methods, section 9.9).

For each ligation mix, the titre was determined as the number of plaque forming units (pfu) per millilitre (Table 6.1). The highest titre observed was in ligation reaction number three which showed a titre of 4.2×10^5 pfu ml^{-1}. This sample was amplified to form the genomic library, which was subsequently screened. After amplification by high density plating, bacteriophage clones were eluted into 3ml phage buffer, at 4°C for sixteen hours. Bacterial cell debris was removed and crystalline NaCl added to a final concentration of 1M to stabilise the recombinant phage. The amplified library was re-titred before storage at 4°C with $CHCl_3$. After amplification the library showed a titre of 7.0×10^{10} pfu ml^{-1} and was calculated as containing 420 genome equivalents per ml, with an average DNA insert of 15Kb.

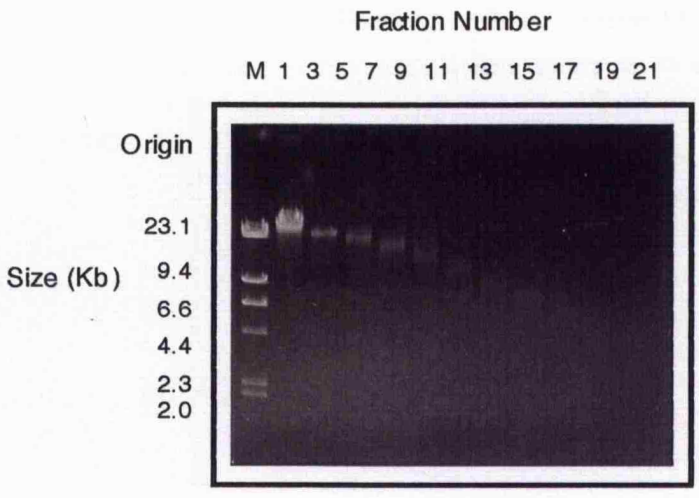


Figure 6.1: *Sau*3A partial digests of S288C a genomic DNA. 25μl Sucrose gradient fractions were analysed by agarose gel electrophoresis on a 0.5% (w/v) agarose gel in 1x TAE buffer. M is a size marker *Hind*III cut lambda DNA. The size marker DNA was suspended to the same sucrose concentration as that of the gradient samples. Fractions analysed were alternate from fraction number one.

Table 6.1: Ligation reaction composition. Ligation reactions were in a total volume of 5.0µl, with varying concentration of genomic insert DNA. All figures shown in the table below indicate volume in µl. P/C is the positive control using 16Kb DNA fragment (Promega) and Background contains no insert DNA. The number of plaque forming units obtained per ligation reaction after packaging are indicated in the table below.

	Ligation Reaction					
	1	2	3	4	P / C	Background
Vector DNA (0.5 µg/µl)	1.0	1.0	1.0	1.0	1.0	1.0
Insert DNA 100 ng/µl	2.0					
50 ng/µl		2.0	1.0			
10 ng/µl				1.0		
Positive (100 ng/µl)					1.0	
P.F.U / ml (*10^5)	0.4	0.9	4.2	0.6	13.0	0.0

6.3 Isolation of λ clones containing *ESP30* gene

The amplified genomic library was screened by *in situ* hybridization of bacteriophage DNA immobilised on nitrocellulose filters using the radiolabelled cDNA clone as probe DNA (Maniatis *et al.*, 1982). Plaques showing a positive hybridization signal, were isolated and purified by repeated 'picking', re-plating and screening, until a homogeneous signal population was obtained ('plaque purification'). After the tertiary screen, homogeneous signal populations were produced and single plaques were isolated, which were used to prepare bacteriophage DNA for subsequent analysis.

In the initial primary screen, thirty five individual clones were isolated. Based upon the strength of their hybridization signal to the *ESP30* cDNA probe, two classes of clone were identified; one class exhibited a 'strong' and one a 'weak' hybridization signal (under high stringency hybridization conditions). Bacteriophage clones which showed a 'strong' hybridization signal were λ^5 , λ^{14} , λ^{28} , λ^{31}, whereas clones λ^{15}, λ^{26} , λ^{27}, λ^{35} gave a 'weak' hybridization signal. All were then taken through two further rounds of 'plaque purification' until homogeneous populations were achieved. Individual clones are indicated as their primary plaque number; i.e. λ^{14} was the fourteenth plaque which hybridized with *ESP30* cDNA clone on the primary screen. After a homogeneous population of bacteriophage clones was isolated, DNA was recovered from each clone and characterized by restriction endonuclease digestion, to size the insert and detect individual members of the *ESP30* multigene family.

6.4 Characterization of individual λ clones

Preliminary characterization of bacteriophage clone DNA was by restriction endonuclease digestion and 'Southern' hybridization analysis in order to locate the region of homology to the *ESP30* cDNA. Bacteriophage clones exhibiting the same restriction endonuclease digestion pattern would be derived from the same locus within the yeast genome. Whereas clones with differing restriction digest patterns, would indicate that they were probably derived from different chromosomal loci.

Bacteriophage DNA (2.0μg) extracted from each clone was digested with the individual restriction endonucleases *Bam*HI, *Eco*RI and *Hin*dIII. Restriction digest patterns showed that three unique clones had been isolated, λ^{14} , λ^{15} and λ^{27}, where the cDNA was localised to an approximate three kilobase region in clones λ^{14} and λ^{15} and to within a 5.8 kilobase region in λ^{27} (figure 6.2). None of the clones

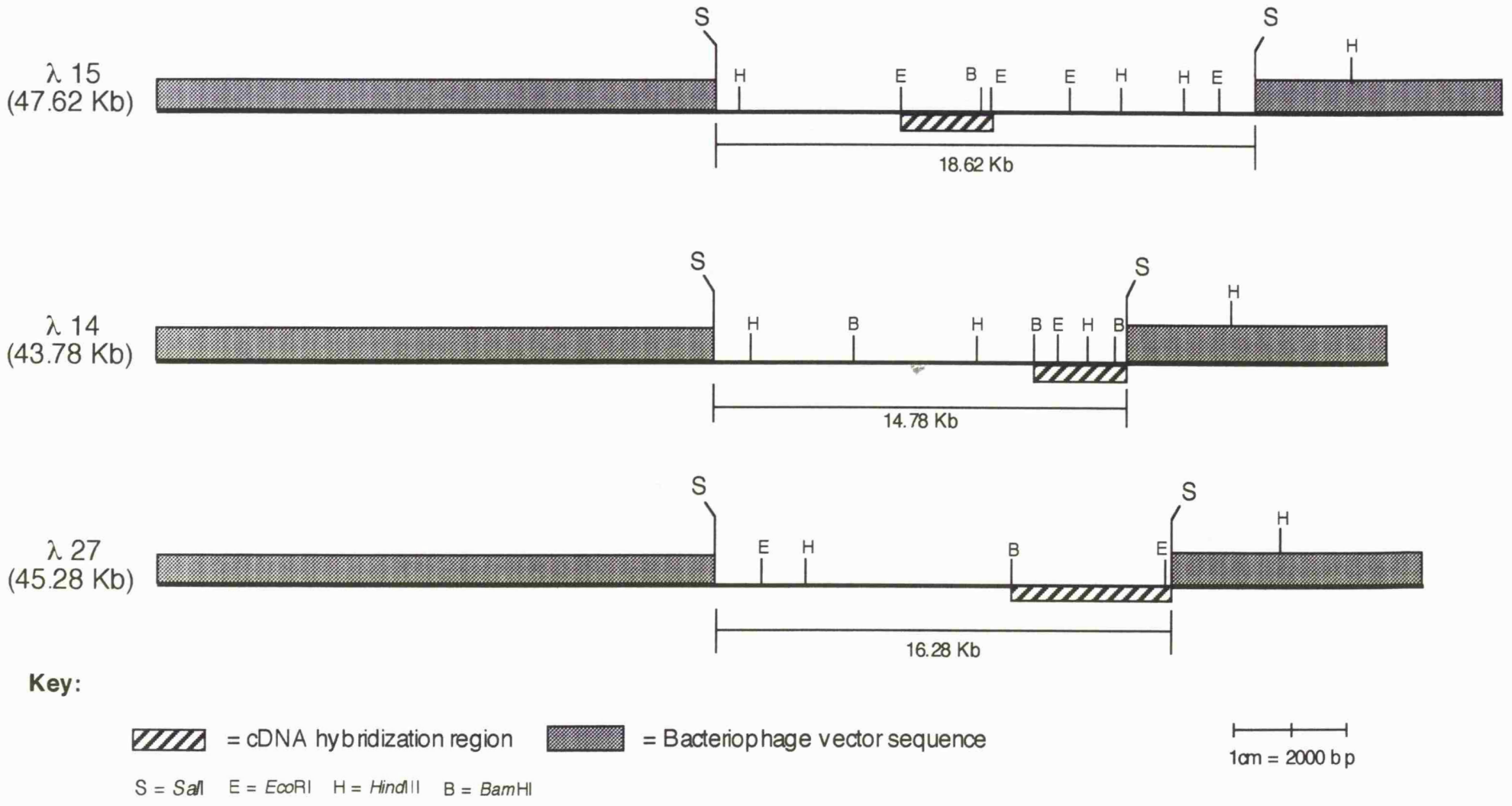


Figure 6.2: Restriction endonudease map of λ clones 14, 15 and 27. Partial restriction map of λ14 ('strong' primary hybridization signal), λ15 ('weak' primary hybridization signal) and λ27 ('weak' primary hybridization signal) with *Bam*HI, *Eco*RI and *Hind*III. Restriction digestion of 2.0μg λ DNA with 10u restriction endonudease was in a total reaction volume of 20μl at 37°C for two hours. 'Southern' blot was hybridized with *ESP30* cDNA clone under non-permissive conditions. Size of DNA fragments was determined by comparison of migration distance vs molecular weight of known DNA size marker. Length of individual genomic DNA insert is indicated in kilobase pairs (Kb).

isolated were overlap clones for λ^{15} or λ^{14}, however, λ^{27} was found to be identical to λ^{28}. A limited restriction endonuclease map showed that the individual clones λ^{14}, λ^{15} and λ^{27} were different. Because of the difficulties in handling several clones simultaneously, the remaining λ clones; λ^{5} , λ^{26}, λ^{31} and λ^{35} were not classified by restriction endonuclease digestion and 'Southern' hybridization analysis.

'Southern' hybridization with the *ESP30* cDNA clone, under high stringency conditions showed a single hybridizing fragment from each λ clone, which corresponded to the same size of fragment detected by hybridization of *ESP30* cDNA to genomic DNA cut with the same restriction endonuclease (figure 6.3). *Eco*RI cut λ^{14} DNA showed a single hybridization signal at 9.3Kb, λ^{15} at 3.2Kb and λ^{27} at 4.0Kb (each hybridization signal is marked by a black triangle, see figure 6.3), which corresponded to fragments detected in a genomic DNA 'Southern' hybridization (see chapter five) and thus indicated that three different loci from the yeast genome had been isolated.

6.5 Isolation of fragments into pUC vectors.

In the subsequent stages, in the characterization of the identified *ESP30* family members, plasmid vectors were used as these allowed easier manipulation and handling than the original bacteriophage clones. Convenient restriction endonuclease fragments of the lambda clones λ^{14} and λ^{15} were subcloned into pUC based vectors; the clone λ^{27} was not taken further in this study. In the two distinct bacteriophage clones, λ^{14} and λ^{15}, the cDNA binding regions were delimited to fragments approximately 3.0Kb in size. Because certain restriction endonuclease sites located within the cDNA homologous region were used in the subsequent cloning strategies, it was necessary to construct two separate plasmid clones for each λ clone, thus ensuring that this region would be isolated. Individual lambda clones were digested with appropriate restriction endonucleases to produce DNA fragments in the size range 3.0-6.0Kb. λ^{14} was digested with the individual restriction endonucleases *Bam*HI and *Hin*dIII, to release fragments 3.2Kb and 6.3Kb, respectively, which were cloned into pUC8 and the resultant plasmids termed pRH3 and pRH8, respectively. In contrast λ^{15} DNA was digested with *Eco*RI and *Xho*I to release fragments 3.2Kb and 3.8Kb, respectively. These fragments were sub-cloned into the plasmid vector pUC18 on appropriate restriction sites and the resultant plasmids termed pRH4 and pRH11, respectively. Initial characterization of these clones involved the determination of a detailed restriction endonuclease map for each clone (see appendix two) and 'Southern' hybridization with the *ESP30* cDNA clone, in order to show the overlap region, which bridges the cDNA binding region (figure 6.4).

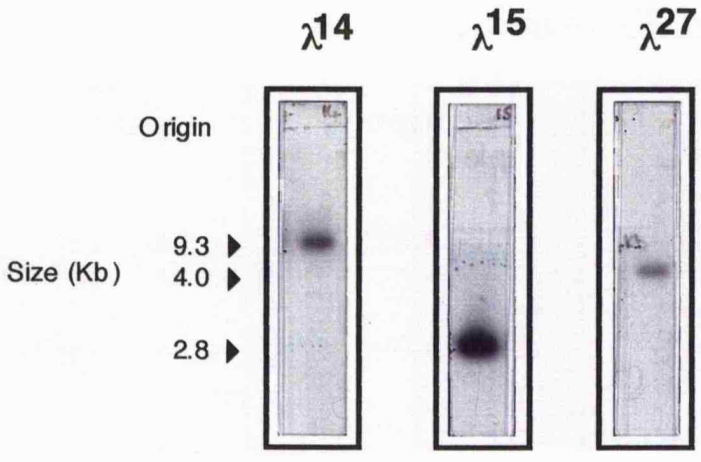


Figure 6.3 : 'Southern' hybridization lambda 14, 15 and 27 bacteriophage clones. Lambda DNA (2.0μg) from bacteriophage clones 14, 15 and 27, cut with 10u *Eco*RI in a total reaction volume of 20μl at 37°C for two hours, followed by hybridization with *ESP30* cDNA clone under non-permissive conditions, indicated a single hybridization band per clone, corresponding to the same size of genomic fragment detected in a total genomic 'Southern' hybridization (see text).

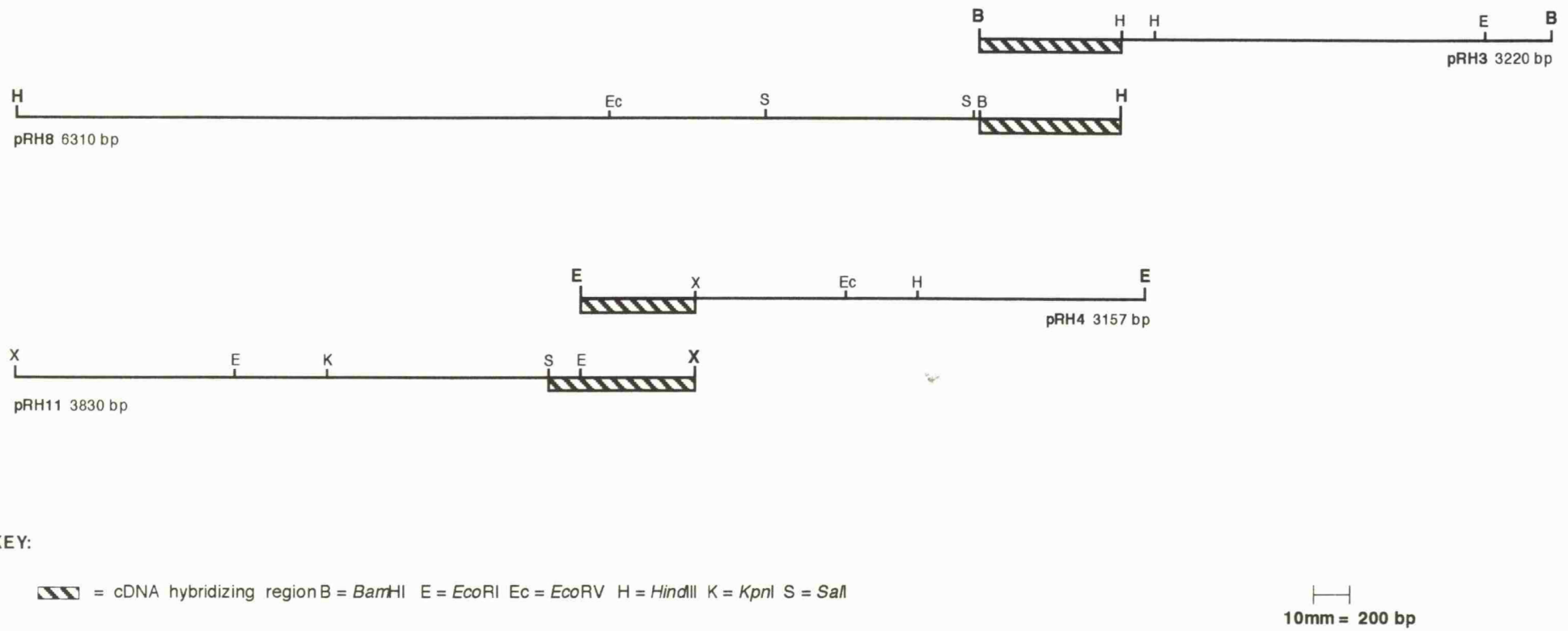


Figure 6.4: Restriction endonuclease map of lambda clone fragments in a pUC vector. Lambda clone fragments were sub-cloned into pUC18. Restriction endonuclease map of individual fragments from lambda clones 14 and 15 show the size of the fragment inserted, the main restriction sites (restriction sites used in the sub-cloning are shown in bold-type, other sites are also shown for comparison with the individual lambda clone) and the designation of the plasmid. The cDNA binding region is also indicated, as is the overlap between the different clones spanning that region.

6.6 Construction of nested deletions in plasmid clones pRH3/8 and pRH4.

In order to sequence DNA fragments greater than 1.0Kb in length, a series of nested deletions can be constructed. Nested deletions as the name implies are a series of deletions within the target DNA, which extend along the length of the DNA to bring 'new' regions into the sequencing range of the primer (Henikoff, 1984; Henikoff, 1987). Deletions are produced by the enzymatic digestion of the target DNA in a plasmid vector, which can then be recircularized to generate functional plasmids without the need for another intermediate cloning stage. Exonuclease III, a 3' exonuclease specific for double stranded DNA, initiates unidirectional digestion of the target DNA from either a recessed 3' end or a blunt end, but not a 3' single strand extension. After unidirectional digestion of the target DNA, for varying lengths of time, the generated 5' single strand is removed by digestion with S1 nuclease or exonuclease VII and the plasmid recircularized, prior to transformation into a suitable *E. coli* host strain. Suitable transformants are then screened for by 'mini-prep' DNA analysis with appropriate restriction endonucleases. Incomplete DNA sequence coverage between progressive nested deletions can then be covered by oligonucleotide primer walking (Strauss *et al.*, 1986).

The most appropriate DNA sequencing strategy for the analysis of DNA fragments from bacteriophage clones λ^{14} and λ^{15} in pUC based vectors was the construction of a series of nested deletions by exonuclease III, followed by dideoxy sequencing of the individual deletion clones. Restriction endonuclease combinations used to generate sites suitable for unidirectional exonuclease III treatment of plasmid clones pRH3 and pRH8 (λ^{14}) were *Hinc*II/*Pst*I and*Xba*I/ *Kpn*I, respectively. Plasmid clone pRH4 (λ^{15}) was digested with *Hinc*II and *Pst*I. In each case 5.0µg CsCl density gradient purified plasmid DNA (see Materials and Methods) was digested with 20u of each restriction endonuclease.

Digested plasmid DNA was ethanol precipitated, followed by exonuclease III digestion (190u per 2.5µg DNA) at 37°C for ten minutes. Samples were taken every minute and the reaction terminated by the addition of EDTA. The single 3' extension DNA strand was removed by exonuclease VII (2.0u per 0.6µg DNA) digestion at 37°C for sixty minutes and 'blunt' ends formed by a 'fill-in' reaction (materials and methods). The extent of deletion was assessed by agarose gel electrophoresis of 0.2µg plasmid DNA (figure 6.5). Plasmids were then recircularized by incubation with T4 DNA ligase at 15°C and transformed into the *E. coli* host strain DH5α F'. Recombinant clones were selected by ampicillin resistance and a series of progressive

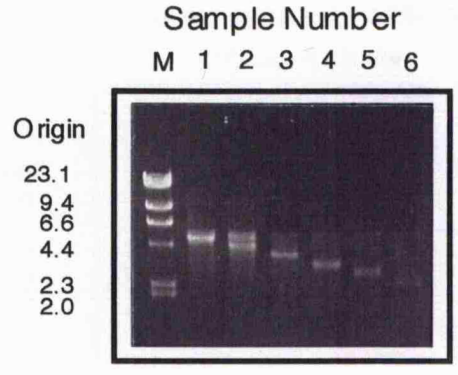


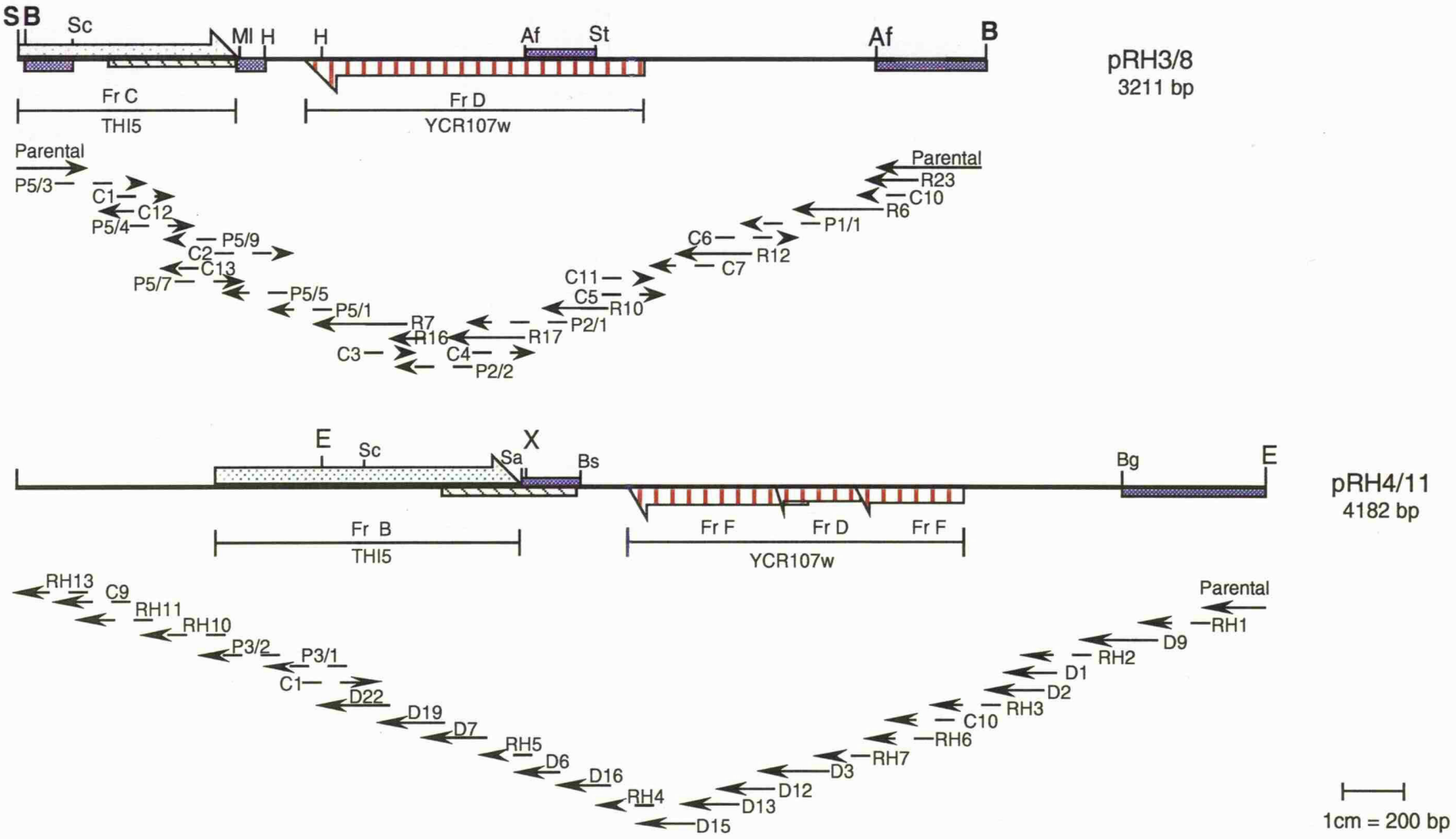
Figure 6.5: Progressive deletions of pRH3 by exonudease III (*Exo*III) digestion. CsCl purified plasmid pRH3 digested with *Pst*I and *Hind*I to form the necessary 3' and 5' termini for *Exo*III digestion (see text for details). The progressive deletions in the plasmid over a twelve minute incubation period can be clearly seen. Where M is a known DNA size standard (*Hind*III cut lambda DNA) and individual sample points are indicated corresponding to incubation at 37°C accumulating every two minutes upto sample six (twelve minutes after addition of *Exo*III).

deletions for each plasmid clone identified by restriction endonuclease mapping. Clones which showed an approximate 200-300 base progressive invasion in the extent of the deletions were used in subsequent DNA sequence analysis.

Plasmid DNA was isolated using a modified alkaline lysis protocol (Birnboim and Doly, 1979), from an overnight 50ml culture of the deletion clone grown under antibiotic selection. The M13/pUC universal reverse sequencing primer (rev -40) was used in all sequence reactions with the plasmid deletion clones. DNA sequence obtained using the T7 DNA sequencing protocol usually resulted in a sequence fidelity of between 250-350 resolved bases. Gaps in the DNA sequence between individual deletion clones, were bridged by primer walking from the previous deletion clone. The individual oligonucleotide primers used in the sequencing reactions are shown in appendix one (see also figure 6.6). Each plasmid clone was initially sequenced in one direction only, with regions of ambiguity sequenced in both directions.

The final consensus DNA sequence of each plasmid clone was compiled by manual alignment of individual contiguous sequences with LINEUP (Devereux, Haeberli and Smithies, 1984). DNA sequence from the deletion clones of plasmids pRH3 and pRH8 were combined to form the consensus sequence RH3/8 (3211nt) (appendix three). Deletion clones of pRH4 and DNA sequence obtained from pRH11 by primer walking were combined to form the consensus sequence RH4/11 (4182nt) (appendix four & EMBL accession no. Z48220). The integrity of these DNA consensus sequences was assessed by the comparison of the restriction endonuclease map for each plasmid clone (appendix two) compared to the predicted endonuclease sites within the DNA sequence. The predicted pattern of restriction endonuclease sites matched those obtained by experimental digest. However, in the RH3/8 consensus, DNA sequence above 3211nt was identified as the left arm of the λ EMBL3 vector (data not shown). From examination of the DNA sequence, it was apparent that the initial *Sau*3A partial digestion of the original λ^{14} clone had resulted in the formation of a *Bam*HI site. This clone had been originally mapped to an *Eco*RI generated genomic DNA fragment of 9.3Kb, however, this was due to the inclusion of the vector fragment and not a result of the cloned DNA. A schematic representation of the RH3/8 and RH4/11 consensus sequences is shown in figure 6.6. The extent and direction of the DNA sequence for each of the individual deletion clones of pRH3 and pRH4 is shown, with individual primers (appendix one) used to bridge between adjacent deletion clones also indicated (figure 6.6).

Figure 6.6: Schematic diagram of the plasmid clones RH3/8 and RH4/11 DNA sequence. Deletion clones and oligonucleotide primers used in the DNA sequence strategy and the direction of sequencing are shown as arrows. Sequence of the oligonucleotide primers is shown in appendix one. The largest open reading frames are indicated as shaded arrows. Restriction fragments used in the analysis of gene function (see text for details) and the *ESP30* cDNA binding region are indicated as shaded boxes. Important restriction endonuclease sites are also shown.



KEY:

- = Oligonucleotide primer
- = Deletion clone
- = Restriction endonuclease fragment
- = Proposed open reading frame. Arrow indicates direction of ORF transcription.
- = De-limited ESP30 cDNA hybridization region

Figures indicate the following: Rxx, Dxx = Deletion clone label
Cxx, Pxx, RHxx = Primer designation
Fr xx = Open reading frame

Restiction enzymes shown are as follows: Af *Afl*III, B *Bam*HI, Bg *Bgl*I, Bs *Bsg*I, E *Eco*RI, H *Hind*III, Ml *Msl*I, S *Sal*I, Sa *Sap*I , Sc *Sca*I, St *Stu*I and X *Xho*I.

6.7 Analysis of the RH3/8 and RH4/11 consensus sequences

Initial characterization of these two consensus sequences involved their comparison to already published sequence data deposited in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database. A FASTA type search showed the RH3/8 DNA sequence to be contained within a 8.2Kb sequence published by A. Goffeau (EMBL accession No. X83226) (van Dyck, Pascual-Ahuir and Goffeau, 1995). The Goffeau sequence covers the entire *ESP30* hybridization region, which corresponds to an 2.8Kb *Eco*RI fragment (see chapter five) and extends 1199 nucleotides 5' to the start of the RH3/8 consensus sequence and 3813 nucleotides 3' (figure 6.7). The only difference between the Goffeau and RH3/8 consensus sequences is a single nucleotide omission (C) at position 2854nt, in the RH3/8 DNA consensus, with the corresponding residue at position 4051nt in the Goffeau sequence.

Open reading frames (ORF's) within the RH3/8 consensus sequence were identified by the translation of the DNA sequence in all six frames, using the MAP package (Devereux *et al.*, 1984). The largest intact open reading frames detected (figure 6.6), corresponded to two separate proteins, with a predicted size of 349 and 225 amino acid residues in length, respectively, where this latter open reading frame spanned the *ESP30* cDNA hybridization region (figure 6.6). The corresponding Goffeau sequence which spans the same region, contains a complete open reading frame of 340 amino acids in length (figure 6.7). Therefore, the pRH3 and pRH8 plasmid clones lack the promoter and part of the coding region for this gene.

The open reading frame identified in RH3/8 of 225 amino acid residues in length, spanned the *ESP30* cDNA binding region (249 to 794nt)) and showed an overall 53.8% identity to the thiamine regulated gene *nmt1a* in *S. pombe* (figure 6.8a). TFASTA comparison to the same open reading frame of 340 amino acid residues, from the Goffeau sequence showed a 62.0% identity to *nmt1a S. pombe*. As this identified polypeptide was similar to the thiamine regulated gene *nmt1a*, the gene encoding the polypeptide was renamed *THI5* (for THIamine regulated 5) (*ESP30*). TFASTA analysis of the remaining open reading frame of 349 amino acids, indicated a 78.5% identity to the 'YCR107w' ORF of *S. cerevisiae* chromosome III (figure 6.9a). A hybridization signal was not detected by 'Northern' analysis, of mRNA isolated from blackstrap molasses grown cells (see Chapter two, section 2.2.2), when probed with the 'YCR107w' open reading frame from pRH3 (data not shown).

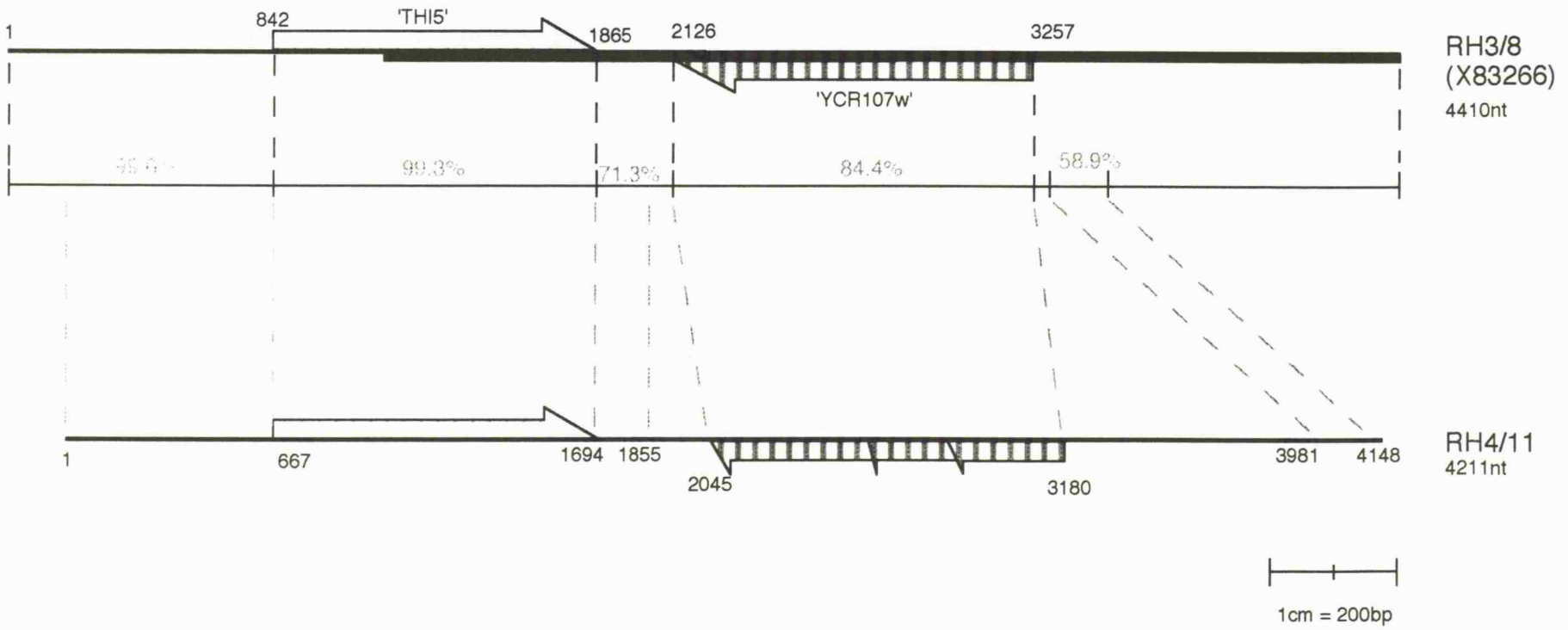
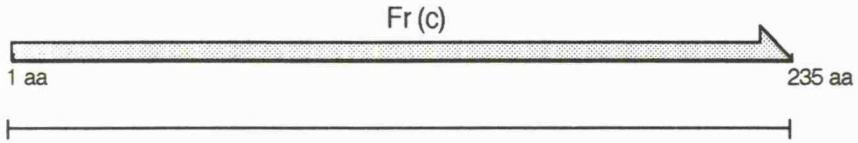


Figure 6.7: Percentage identity of RH3/8 vs RH4/11 consensus DNA sequence. Open reading frames are indicated asshadede arrows. Dashed lines indicate the areas of identity. Figures in black indicate the sequence position, figures in red show the percentage identity. Solid black line shows DNA sequence, double thickness line indicates RH3/8 DNA sequence.

Figure 6.8: GAP analysis of the inferred open reading frames in RH3/8 and RH4/11 against the thiamine regulated gene *nmt1a* in *S. pombe*. The orientation of the inferred open reading frames is indicated as shaded arrows and the percentage similarity is shown below the open reading frames; **A:** RH3/8 open reading frame **B:** RH4/11 open reading frame. Similarity between the individual amino acids is shown, where ' | 'is an exact match, ' : ' is a conserved function and ' . ' indicates a neutral substitution.

A

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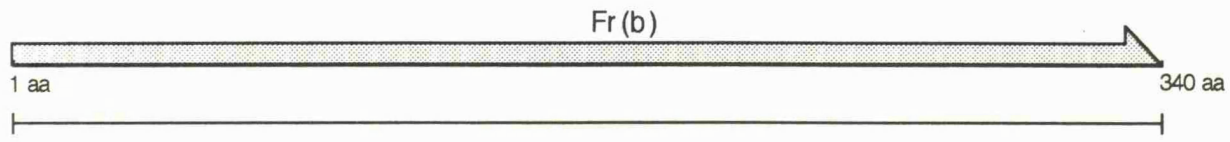
RH38(c) ..............EFGKIQIDELTKHYGMKPEDYTAVRCGMNVAKYIIEGKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLACLGCCCFCTV
                      ||||||:|:|...:|:.|.||||:|||||:|. ||:|.||:|||||||||||||| : ..|||| ||..|||||:|| ||||||||:
 SPNMT1 DFKDIKGKRIGYVGEFGKIQLDDLCSKFGLSPSDYTAIRCGMNIAPAIINGEIDGGIGIECMQQVELERWCVSQGRPRSDVQMLRIDRLANLGCCCFCTI

RH38(c) LYICNDEFLKKNPEKVRKFLKAIKKATDYVLADPVKAWKEYIDFKPRLNNDLSYKQYQRCYAYFSSSLYNVHRDWKKVTGYVRDLEEGYRLRKRLAILPP
        |||.:|||: |:|:|:: ||:||..||  :| |||..:||||.||. :..:|  .|::||:|||| .: ||.|||.|||.|          .|||:|:|.
 SPNMT1 LYIAHDEFIAKHPDKIKAFLRAIHSATLDMLKDPVQTYKEYIHFKREMGSELHREQFERCFAYFSHDISNVPRDWNKVTNY.........SKRLGIIPQ

RH38(c) DYVSNYTNEYLSWPEPEEVSDPLEAQRLMAIHQEKCRQEGTF.....KRLALPA*
        |: .|:||:||.|. ..: .||:: |  :|  |:..:|.|..      |.. ||
 SPNMT1 DFEPNCTNGYLTWELDPDEKDPMGKQEAIAEIQDEIKQKGGVFSGNSLRYVEPANL*
```

Percent Similarity: 71.111 Percent Identity: 54.667

B

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RH411(b) MSTDKITFLLNWQPTPYHIPIFLAQTKGYFKEQGLDMAILEPTNPSDVTELIGSGKVDMGLKAMIHTLAAKARGFPVTSVASLLDEPFTGVLYLKGSGIT
         |||:||||| ||::||||:|||||||:||:. :|:::|||||||||||.|||||||||||||||||||||:||||.:|||:|||||:: |||.||
  SPNMT1 MSTNKITFLTNWEATPYHLPIFLAQTRGYYEREGIEVAILEPTNPSDVTALIGSGKVDMGLKAMIHTLAAKARGYPVTSFGSLLNEPFTGLITLKGNGI.

RH411(b) EDFQSLKGKKIGYVGEFGKIQIDELTKHYGMKPEDYTAVRCGMNVAKYIIEGKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLACLGCCCFCT
         :||..:|||:||||||||||:|:|...:|:.|.||||:|||||:|. ||:|.||:|||||||||||||| : ..|||| ||..|||||:|| ||||||||
  SPNMT1 NDFKDIKGKRIGYVGEFGKIQLDDLCSKFGLSPSDYTAIRCGMNIAPAIINGEIDGGIGIECMQQVELERWCVSQGRPRSDVQMLRIDRLANLGCCCFCT

RH411(b) VLYICNDEFLKKNPEKVRKFLKAIKKATDYVLADPVKAWKEYIDFKPQLNNDLSYKQYQRCYAYFSSSLYNVHRDWKKVTGYGKRLAILPPDYVSNYTNE
         :|||.:|||: |:|:|:: ||:||..||  :| |||..:||||.||.:::..:|  .|::||:|||| .: ||.|||.|||.|:|||:|:|.|: .|:||:
  SPNMT1 ILYIAHDEFIAKHPDKIKAFLRAIHSATLDMLKDPVQTYKEYIHFKREMGSELHREQFERCFAYFSHDISNVPRDWNKVTNYSKRLGIIPQDFEPNCTNG

RH411(b) YLSWPEPEEVSDPLEAQRLMAIHQEKCRQEGTF.....KRLALPA* 340
         ||.|. ..: .||:: |  :|  |:..:|.|..      |.. ||
  SPNMT1 YLTWELDPDEKDPMGKQEAIAEIQDEIKQKGGVFSGNSLRYVEPANL* 346
```

Percent Similarity: 78.761 Percent Identity: 61.947

Restriction endonuclease analysis of the plasmid clones pRH4 and pRH11 has shown that these clones are distinct from those of pRH3 and pRH8. FASTA analysis showed the RH4/11 DNA consensus sequence as 96.9% identical over 1863 nucleotides to the RH3/8 (X83226) DNA sequence (figure 6.7). In the RH4/11 consensus, a region of homology was identified, which corresponded to the *THI5* gene found on RH3/8, which covered the *ESP30* hybridization region. This RH4/11 *THI5* region showed a 99.5% homology upto 1694nt to the RH3/8 (X83226) *THI5* region (figure 6.7). 3' to this region, which covers the 'YCR107w' region of homology, the level of DNA similarity decreases, where in the RH4/11 consensus, a region 2121 to 3352nt, showed an 80.0% identity to the *S. cerevisiae* 'YCR107w' gene chromosome III (312,608 to 313,699nt), whereas the corresponding region in RH3/8 consensus showed a 80.5% identity over 1074 nucleotides (2050 to 3124nt RH3/8) to the 'YCR107w' ORF. This change in similarity of the DNA sequences is discussed below. The 'YCR107w' ORF on chromosome III, shows some identity to an aryl-alcohol dehydrogenase (from *Phanerochaete chrysosporium*) (55.8% identity in 360 amino acid residues).

Translation of the RH4/11 consensus sequence revealed that the predicted open reading frames spanning the 'YCR107w' region of identity, showed three frame shifts, in frames E and F (figure 6.9b), which would result in premature termination of the polypeptide upon transcription/translation. Therefore, this homologue to 'YCR107w' would appeared not to be able to produce a functional protein. Another open reading frame, 340 amino acid residues in length, which also spans the *ESP30* cDNA hybridization region (674 to 1696 nucleotides (appendix four)) was identified as 61.9% identical to the *S. pombe nmt1* gene (figure 6.8b). Comparison of this open reading frame with that of the *THI5* ORF (RH3/8 and X83226 - A. Goffeau), showed a single neutral amino acid substitution; arginine to glutamine (R→Q) in the predicted primary amino acid sequence. This substitution was a result of a single nucleotide alteration (G) at position 387nt RH3/8 when compared to RH4/11 (figure 6.10). Within this coding region six other base changes were detected between the RH3/8 and RH4/11 consensus sequences, however, these mutations did not alter the predicted primary amino acid sequence.

Figure 6.9: Alignment of RH3/8 and RH4/11 'YCR107w' open reading frames with the polypeptide 'YCR107w' chromosome III. Frame shifts in the 'YCR107w' open reading frame in RH4/11 is as indicated in figure 6.6.

A: 'YCR107w' open reading frame (d) on plasmid clone pRH3.

B: 'YCR107w' open reading frame (d) and (e) on plasmid clone RH4/11.

The percentage similarities between 'YCR107w' and the RH3/8 and RH4/11 peptides is as indicated. The alignment of the individual amino-acids in each open reading frame to 'YCR107w' peptide is indicated as ' I ' as an exact match, ' : ' is a conserved substitution. ' . ' indicates a neutral substitution and ' * ' indicates a termination codon.

A:

```
YCR107      MIGSASDSSSKLGRLRFLSETAAIKVSPLILGEVSYDGARSDFLKSMNKNRAFELLDTFYEAGGNFIDAANNCQNEQSEEWIGEWIQSRR
RH38.D      MTDLFKPLPEPPTELGRLRVLSKTAGIRVSPLILGGASIGDAWSGFMGSMNKEQAFELLDAFYEAGGNCIDTANSYQNEESEIWIGEWMASRK

YCR107      LRDQIVIATKFIKSDKKYKAGESNTANYCGNHKRSLHVSVRDSLRKLQTDWIDILYVHWWDYMSSIEEFMDSLHILVQQGKVLYLGVSDTPAW
RH38.D      LRDQIVIATKFTGDYKKYEVGGGKSANYCGNHKRSLHVSVRDSLRKLQTDWIDILYIHWWDYMSSIEEVMDSLHILVQQGKVLYLGVSDTPAW

YCR107      VVSAANYYATSYGKTPFSIYQGKWNVLNRDFERDIIPMARHFGMALAPWDVMGGGRFQSKKAMEERRKNGEGIRSFVGASEQTDAEIKISEAL
RH38.D      VVSAANYYATSHGKTPFSVYQGKWNVLNRDFERDIIPMARHFGMALAPWDVMGGGRFQSKKAMEERRKNGEGLRTFVGGPEQTELEVKISEAL

YCR107      AKIAEEHGTESVTAIAIAYVRSKAKNFFPSVEGGKIEDLKENIKALSIDLTPDNIKYLESIVPFDIGFPNNFIVLNSLTQKYGTNNV*
RH38.D      TKIAEEHGTESVTAIAIAYVRSKAKNVFPLIGGRKIEHLKQNIEALSIKLTPEQIEYLESIVPFDVGFPKSLIGDDPAVTKKLSPLTSMSARI

RH38.D      AFDN*

SCORES:     Frame: (D) 78.5% identity in 349 aa overlap
```

B:

```
YCR107      MIGSASDSSSKLGRLRFLSETAAIKVSPLILGEVSYDGARSDFLKSMNKNRAFELLDTFYEAGGNFIDAANNCQNEQSEEWIGEWIQSRR
RH411.D     MADLFAPAPEPSTELGRLRVLSKSAGIRVSPLILGGMSIGDAWSEILGSMSKERAFELLDAFYEAGGNFIDTANNYQNEQSEAWIGEWMVSRK

YCR107      LRDQIVIATKFIKSDKKYKAGESNTANYCGNHKRSLHVSVRDSLRKLQTDWIDILYVHWW-YMSSIEEFMDSLHILVQQGKVLYLGVSDTPAW
RH411.D     LRDQIVIATKFTTDYKKYDVWWW*:                                                GKVLYLGVSDTPAW
RH411.E                              GGGKS-ANYCGNHKRSLHVSVRDSLRKLQTDWIDILYVHWWDYMSSIEEVMDSLHILVQQ

YCR107      VVSAANYYATSYGKTPFSIYQGKWNVLNRDFERDIIPMARHFGMALAPWDVMGGGRFQSKKAMEERRKNGEGIRSFVGASEQTDAEIKISEAL
RH411.D     VVSAANYYAKSHGKTPFSIYQGKWNLLNRDFERDIIPMARHFGMALAPWDVMGGGRFQSKKAMEERRKNGEGIRSFVGASEQTDAEIKISEAL

YCR107      AKIAEEHGTESVTAIAIAYVRSKAKNFFPSVEGGKIEDLKENIKALSIDLTPDNIKYLESIVPFDIGFPNNFIVLNSLTQKYGTNNV*
RH411.D     AKVAEEHGTESVTAIAIAYVRSKAKNVFPLVGGRKIEHLKQNIEALSIKLTPEQIKYLESIIPFDVGFPTNFIGDDPAVTKKASLLTAMSAQISFD*

SCORES:     Frame: (E) 27.3% identity in 289 aa overlap  Frame: (D) 67.9% identity in 352 aa overlap
```

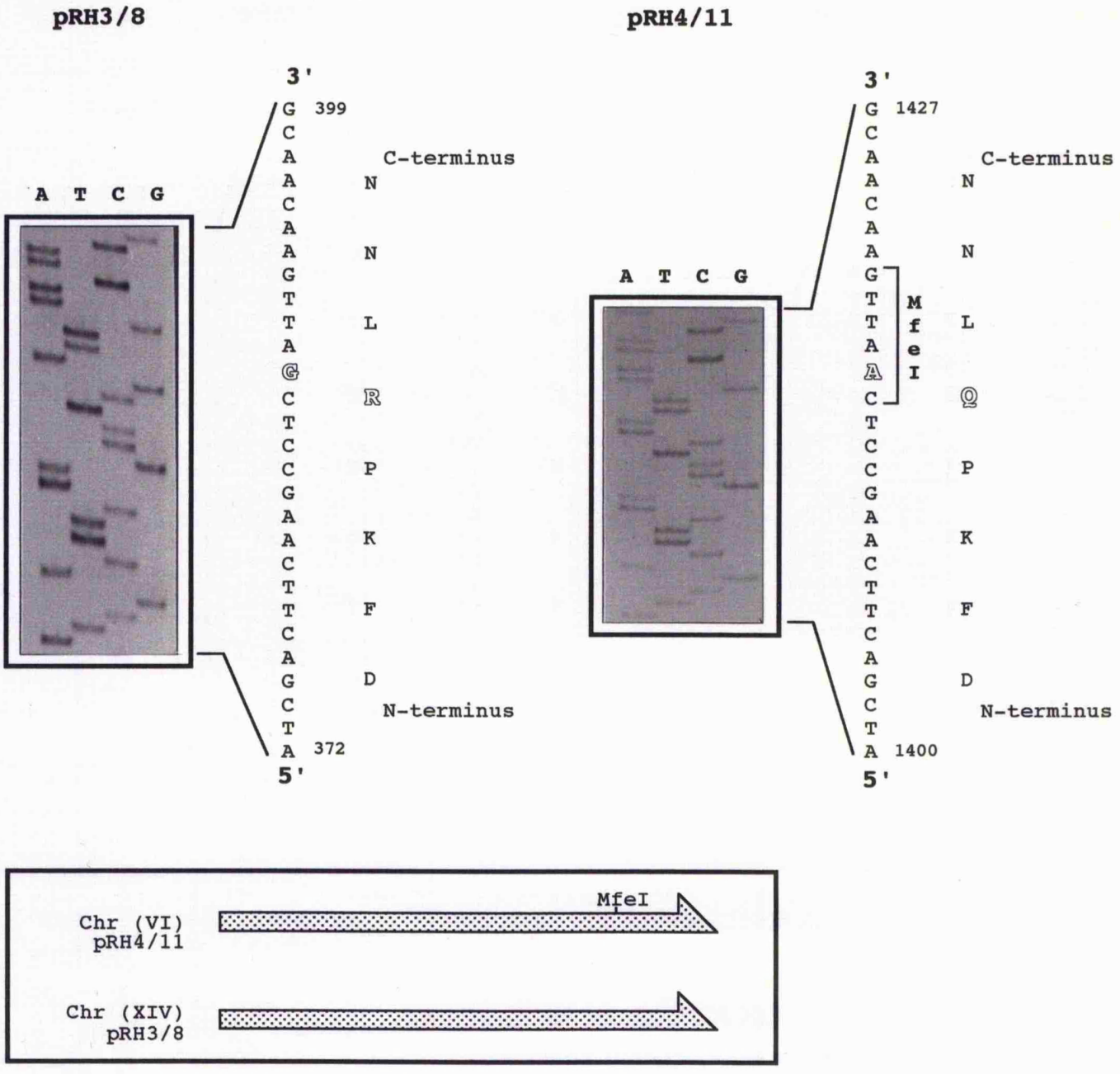


Figure 6.10: Single nucleotide base alteration in the *THI5* coding sequence in chromosome XIV (RH3/8) compared with *THI5* coding sequence chromosome VI (RH4/11). A single nucleotide alteration at base number 387nt in the RH3/8 ORF, results in an conserved amino-acid substitution (R-->Q) when compared with the open reading frame RH4/11 and results in an unique restriction endonuclease site, *Mfe*I. Single nucleotide base alterations and amino-acid substitutions are indicated as shadow, bold text.

As both RH3/8 and RH4/11 showed an open reading frame homologous to the S. *pombe nmt1a* which spanned the *ESP30* cDNA binding region, the question of which gene is transcribed arises. As the mRNA used in the production of the cDNA clone, would have originated from the transcribed gene, the cDNA sequence (see Chapter five) would therefore show a 100% identity to that of the transcribed gene. Comparison of the RH3/8 and RH4/11 DNA sequence to the cDNA sequence (see Chapter five, section 5.3), by FASTA analysis showed that the RH4/11 consensus was 100% identical between 1277 to 1831nt to the cDNA sequence. Whereas, in the RH3/8 clone, the cDNA showed a 93.2% identity over 557 bp to the RH3/8 sequence (249 to 806 bp), with the most divergent region between the cDNA and the RH3/8 sequence in the 3' region of the identified open reading frame (figure 6.11). Therefore, the gene covered by the RH4/11 plasmid clones appeared to contain the functional gene.

6.8 Chromosome location

Pulse-field gel electrophoresis of chromosome DNA molecules, in conjunction with 'Southern' hybridization provides for the easy assignment of a cloned gene to one of the sixteen individual yeast chromosomes. Pulse-field gel electrophoresis separates large DNA molecules by the periodic alternation of electrical fields, with separation based upon the size-dependent retardation of large DNA molecules. A uniform electric field is generated in CHEF (Contour-clamped Homogeneous Electric Field) that alternates between two orientations, which results in the uniform separation and trajectory of the DNA molecules (Chu, Vollrath and Davis, 1986). Yeast strain YPH149 was developed, in which all sixteen yeast chromosomes are resolved (as seventeen linear DNA bands) by CHEF electrophoresis, thus allowing unambiguous chromosome assignment (Gerring, Connelly and Hieter, 1991).

Agarose plugs containing embedded chromosomes of the yeast strain YPH149 were a gift from Dr R. Mount, Biorad Ltd and were used in this study. The chromosomes were resolved by CHEF electrophoresis on a Biorad CHEF-DRII pulsed field system (see Materials and Methods, section 9.12). Resolved chromosomes were transferred onto a nylon support membrane, prior to 'Southern' hybridization with various probe DNA. Chromosome locations were allocated by comparison of the position of the hybridization signal with the migration distances of the stained chromosomes.

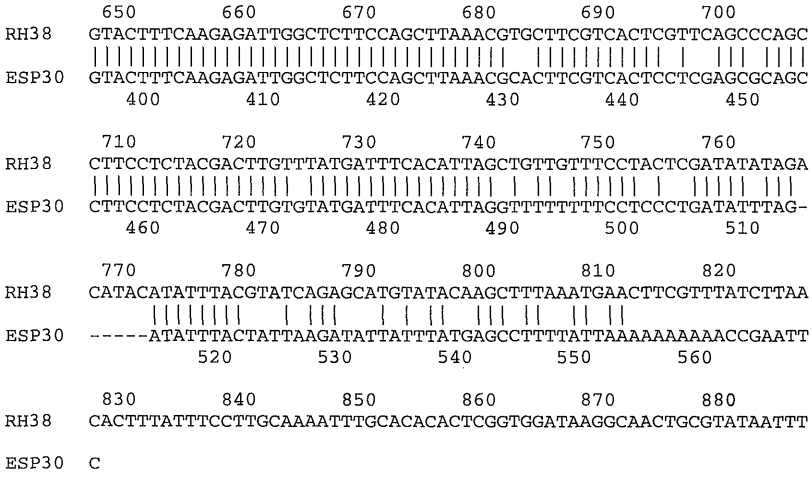


Figure 6.11: *ESP30* cDNA homology to RH3/8 DNA sequence. FASTA analysis of the *ESP30* cDNA to RH3/8 showed a 93.2% identity over 557 bp, exact matches are shown as ' | '. The 3' region of the gene showed the variation between the two sequences and is illustrated below.

The chromosome location for each representive clone was determined, by 'Southern' hybridization of CHEF resolved chromosomes, to restriction endonuclease fragments, containing DNA sequence unique to each individual λ clone; pRH3 (λ^{14}), *Bam*HI/*Afl*III (366bp) and pRH4 (λ^{15}) *Eco*RI/*Bgl*II (483bp) (figure 6.6). From the comparison of migration distances, λ^{14} was localised to chromosome XIV and λ^{15} to chromosome VI (figure 6.12). Restriction endonuclease fragments which contained the identified genes 'YCR107w' and *THI5* genes (pRH3 *Afl*III/*Stu*I (228bp) and pRH3 *Bam*HI/*Sca*I (156bp), respectively (figure 6.6)), were then hybridized. Three separate chromosomes were identified as containing homology to their respective probes, *THI5* was localised to chromosomes VI, X and XIV, whereas 'YCR107w' was localised to chromosomes III, VI and XIV (figure 6.12). Chromosome locations were confirmed by hybridization with the known genes; actin (chromosome VI L) and *LEU2* (chromosome III L) (Mortimer *et al.*, 1989). Four hybridization signals were not detected for the individual *THI5* ORF's as were in a total genomic 'Southern' hybridization (see Chapter five, section 5.4). This was a result of the *ESP30* cDNA probe showing a cross-hybridization signal to the 'YCR107w' gene (data not shown).

6.9 Conclusions

In this chapter, the *ESP30* cDNA sequence has been shown to recognise multiple regions within the yeast genome. Three distinct loci have been isolated from a library of genomic DNA cloned in a bacteriophage vector. Comparison of restriction endonuclease maps indicated that three out of four possible members of the family had been isolated. Each clone was shown to generate a restriction fragment, which hybridized to the cDNA clone, that was identical in size to one of the four fragments detected in a 'Southern' hybridization of genomic DNA digested with the same restriction endonuclease. Clones λ^{14} and λ^{15} were further characterized by sub-cloning the cDNA hybridizing region into pUC vectors, prior to determination of their DNA sequence.

Sequence analysis identified a distinct set of two open reading frames in both lambda clones, one ORF was termed *THI5* (*ESP30*) and showed a similarity to the thiamine regulated gene *nmt1a* of *S. pombe*, the other ORF is homologous to the 'YCR107w' gene (chrIII) of *S. cerevisiae*., where *THI5* and 'YCR107w' are associated together in both lambda clones. Chromosomal identification of these groups, identified the 'YCR107w' region on chromosome III (as reported by (Oliver *et al.*, 1992)), with two other regions of identity on chromosomes VI and XIV. *THI5* (*ESP30*) was located to chromosomes VI and XIV, with another region of identity on chromosome X.

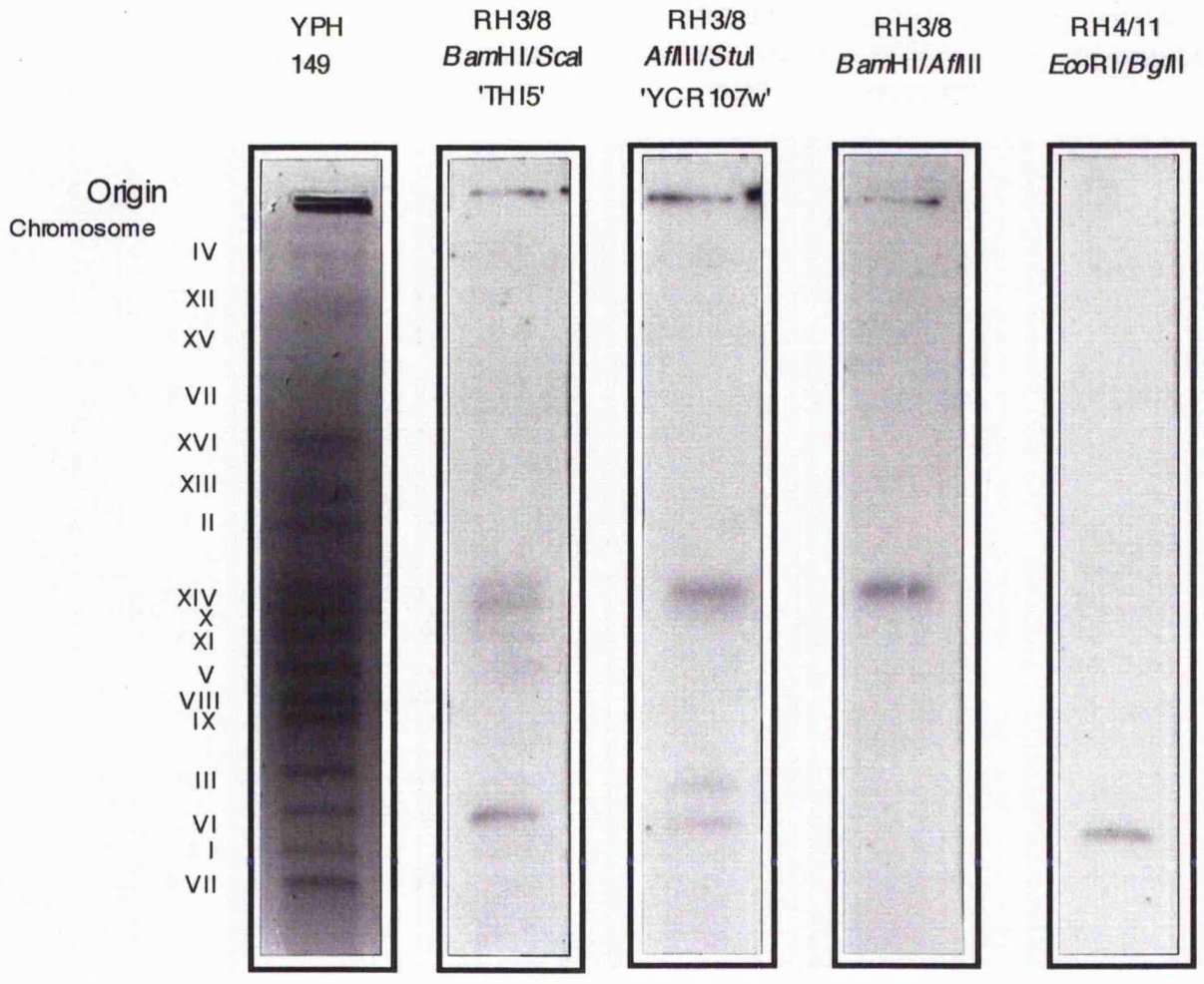


Figure 6.12: Chromosome location of individual lambda clones and *THI5* / *'YCR107w'* homologs. Chromosome locations were assigned to the individual lambda clones by the hybridization of unique restriction endonuclease fragments to separated yeast chromosomes under high stringency conditions. Chromosome separation is shown, hybridization with the fragment RH3/8 *Bam*HI/*Sca*I containing the *THI5* coding region (highly conserved between the clones RH3/8 and RH4/11). The *'YCR107w'* region again was conserved between both series of plasmid clone and was taken from the RH3/8 clone on a *Afl*II/*Stu*I fragment. Unique individual restriction fragments were taken from both clones as indicated in figure 6.6 and text.

The *THI5* (*ESP30*) gene isolated on the plasmid clones RH3/8 (λ^{14}), was incomplete, lacking both the 5' promoter and part of the coding sequence. However, the complete *THI5* sequence representing RH3/8 has been published by A. Goffeau (EMBL Accession No. X83226). FASTA comparison of the *ESP30* cDNA sequence to the RH3/8 DNA sequence (representing the 3' region of the *THI5* coding sequence and untranslated region) showed a 93.2% identity, which indicated that this open reading frame was either not transcribed or is transcribed but had not resulted in the production of the original *ESP30* cDNA clone. However, the RH4/11 (λ^{15}) clones isolated to chromosome VI showed a 100% identity to the *ESP30* cDNA and hence identified the transcribed gene, which had resulted in the production of the original cDNA clone. However, comparison of the RH4/11 (chrVI) and the RH3/8 (X83226) (chrXIV) shows a 99.5% identity between the DNA sequences, which could indicate that these genes have recently been through an recombinagenic event and have yet to diversify. However, this does not appear to be the case as the associated 'YCR107w' region of homology found adjacent to the *THI5* gene, would appear to have diversified, as indicated by the lower degree of DNA identity observed. Another possibility is that the *THI5* (chrXIV) is also functional and is required by the yeast cell for normal growth (see Chapter seven) and hence little diversification has occurred, due to the selective pressure upon this gene locus.

The 'YCR107w' gene product has been identified as showing some identity to a aldoketoreductase and an aryl-alcohol dehydrogenase (Koonin, Bork and Sander, 1994), however, its function has yet to be identified experimentally. In this study, the 'YCR107w' gene has been shown to have identity to three separate regions within the yeast genome; the previously reported chromosome III (Oliver *et al.*, 1992), chromosome VI and XIV. Frame shifts observed in the open reading frame on RH4/11 (chrVI) would result in a truncated polypeptide upon transcription/ translation. The occurrence of multiple copies of this gene would suggest that in the evolutionary past, tandem copies had arisen, allowing divergence of one copy while the other copy retained it's original function. This divergence, however, has resulted in the formation of one possible pseudogene, due to the degree of mutation observed (i.e. the number of frame shifts). The observed multiple copies of the 'YCR107w' and associated *THI5* could be due to the 'YCR107w' gene on chromosome III located in the telomere region which is highly recombinagenic. In conclusion, two copies of the *THI5* (*ESP30*) gene have been sequenced and localised to chromosomes VI, X and XIV, with the proposed functional gene on chromosome VI. The gene 'YCR107w' has been identified as a member of a multigene family with two other family members.

Chapter 7:
THI5 (ESP30) is a thiamine regulated gene possibly involved in the pyrimidine moiety biosynthesis pathway leading to thiamine

7.1 Introduction

Multiple copies of the *THI5* gene have been identified, on chromosomes VI, XIV and a third as yet unclassified locus, on chromosome X (λ^{27}). From published sequence data (September 1995), the chromosome VI locus (*THI5*) has been identified in the left telomere region (12,929 to 13,951nt) and the open reading frame termed 'YFL058W' (Murakami *et al.*, 1995). Transcription of this locus is responsible for the mRNA used in the production of the *ESP30* cDNA clone, which has been shown to be thiamine repressible. The following chapter describes the characterization of this functional gene and the chromosome XIV locus (*THI12*), with a discussion on their function and regulation. The third unclassified locus on chromosome X, will be refered to as *THI11*, in the remaining sections of this thesis. The genes contained within the different loci have been registered within the *Saccharomyces cerevisiae* genome database (SGD) at Stanford, USA and will be used in the remaining chapters of this thesis; *THI5* (chromosome VI), *THI11* (chromosome X) and *THI12* (chromosome XIV).

7.2 Structure of the *THI5* (chrVI) gene

Comparison of the DNA sequence of the *ESP30* cDNA to RH4/11 (λ^{15}) between -663 to +1300nt (figure 7.1 & appendix four) identified a functional *THI5* gene (chapter six). The proposed ATG translation start was at position one, with the coding region +1 to +1020nt and the 5' promoter sequence (-663 to +1nt). Within this 5' region two proposed TATA sequence boxes -40 and -115 bases from the proposed ATG translation start site were identified.

Possible regulatory binding domains were identified in the *Sc. THI5* promoter region by comparison of DNA sequence of the promoter regions from various thiamine regulated genes. Multiple alignment of promoter regions by CLUSTAL V analysis (Higgins *et al.*, 1992), from thiamine regulated genes *THI80*, *THI5*, *THI4* in *S. cerevisiae*, *KMOL1* and *KMOL2* in *K. lactis* and *nmt1a* in *S. pombe*, identified two conserved sequence elements. The first element (5' nGAGCATTCn 3') was located

Figure 7.1: DNA sequence of *THI5*. Complete nucleotide sequence showing the coding region (+1 to +1020nt), 3' un-transiated region (+1020 to +1372nt) and 5' promoter region (-1 to -663nt). The primary amino acid sequence of the open reading frame of 340 amino acid residues encoding a potential protein 38.5 kDa in size is shown in **bold-type** under the DNA sequence. Proposed promoter elements are shown as **bold-type** within the DNA sequence. Proposed TATA sequences are under-lined and the cDNA hybridization region is indicated as shadow type.

Figure 7.1: DNA sequence of *THI5* (chrVI) (-667 to 1562nt)

```
-674 GTAGAACTAGCGATGCTCACACAACGCTAATTTGTTTCCTTCGAAGGGGCAGTCGGCTATTGTAGTTTCTATATATTATGTAGCACAACATGGAAACCATATTCAGCCAGTTTGTATATA
-554 TGAGAATTAAACGTTAAAACTTTTTCATCCTATCAGTCTTTCACATTGGCCTTGCCTAAAGGGCACAATCGTTCACGTATATACATATGTATGTTTACGTATATATATATGTGTTTACAA
-434 ATATGCATGTATATTCTTTTCGAGGAAGATTATTATATGCTATTAGGCGATGAGTCGGCTTAATTTTTTAGGGGGACGAGATTGGCGCGTTAGTGCATAAAGACAGCAAATCACCTGGAG
-314 GTGACAACATTAATGCATTTATCGGTTTTTGGCTATGATGCAATGAGTACAGTTCCAATTGAACGGCTCATGAACTATGAAATATACGAATTATTCAACTAGCAGAAGGCTTGAAATATT
-194 ACTTATTCAGATTATGAAAATGGTGCTGTTATAGCAAAATAAGCAACATCAATCTCTTGCGACGACAGCAAACAAAACATATAAAAACCTCGTATTATCATCTTCATGAACAATATTCTT
- 74 GTCCATTCTTTTGTTCTTTTACCTTTTCAATTTTATAAAAATACCACAACAACAATAAACACACTTCCAAGCTATGTCTACAGACAAGATCACATTTTTGTTGAACTGGCAACCAACCCC
                                                                                                    M  S  T  D  K  I  T  F  L  L  N  W  Q  P  T  P

 194 ATACCATATTCCAATTTTCTTGGCTCAAACCAAAGGTTACTTCAAGGAGCAAGGTCTAGACATGGCCATCCTAGAACCAACCAATCCTTCCGATGTCACTGAGTTAATTGGATCTGGTAA
      Y  H  I  P  I  F  L  A  Q  T  K  G  Y  F  K  E  Q  G  L  D  M  A  I  L  E  P  T  N  P  S  D  V  T  E  L  I  G  S  G  K

 314 GGTCGACATGGGTTTGAAAGCCATGATCCACACCTTGGCTGCCAAGGCCCGTGGTTTCCCAGTGACCTCTGTTGCCTCTTTGTTGGACGAACCATTTACCGGTGTCTTGTACTTAAAGGG
      V  D  M  G  L  K  A  M  I  H  T  L  A  A  K  A  R  G  F  P  V  T  S  V  A  S  L  L  D  E  P  F  T  G  V  L  Y  L  K  G

 434 CAGTGGTATCACTGAAGACTTCCAGTCCCTAAAGGGTAAGAAGATCGGTTACGTTGGTGAATTCGGTAAGATCCAAATCGATGAATTGACCAAGCACTACGGTATGAAGCCAGAAGACTA
      S  G  I  T  E  D  F  Q  S  L  K  G  K  K  I  G  Y  V  G  E  F  G  K  I  Q  I  D  E  L  T  K  H  Y  G  M  K  P  E  D  Y

 554 CACCGCCGTCAGATGTGGTATGAATGTCGCCAAGTACATCATCGAAGGTAAGATTGATGCCGGTATTGGTATCGAATGTATGCAACAAGTCGAATTGGAAGAGTACTTGGCCAAGCAAGG
      T  A  V  R  C  G  M  N  V  A  K  Y  I  I  E  G  K  I  D  A  G  I  G  I  E  C  M  Q  Q  V  E  L  E  E  Y  L  A  K  Q  G

 674 CAGACCAGCTTCTGATGCTAAAATGTTGAGAATTGACAAGTTGGCTTGCTTGGGTTGCTGTTGCTTCTGTACCGTTCTTTACATCTGCAACGATGAATTTTTGAAGAAGAACCCTGAAAA
      R  P  A  S  D  A  K  M  L  R  I  D  K  L  A  C  L  G  C  C  C  F  C  T  V  L  Y  I  C  N  D  E  F  L  K  K  N  P  E  K

 794 GGTCAGAAAGTTCTTGAAAGCCATCAAGAAGGCAACCGACTACGTTCTAGCCGACCCTGTGAAGGCTTGGAAAGAATACATCGACTTCAAGCCTCAATTGAACAACGATCTATCCTACAA
      V  R  K  F  L  K  A  I  K  K  A  T  D  Y  V  L  A  D  P  V  K  A  W  K  E  Y  I  D  F  K  P  Q  L  N  N  D  L  S  Y  K

 914 GCAATACCAAAGATGTTACGCTTACTTCTCTTCATCTTTGTACAATGTTCACCGTGACTGGAAGAAGGTTACCGGTTACGGTAAGAGATTAGCCATCTTGCCACCAGACTATGTCTCGAA
      Q  Y  Q  R  C  Y  A  Y  F  S  S  S  L  Y  N  V  H  R  D  W  K  K  V  T  G  Y  G  K  R  L  A  I  L  P  P  D  Y  V  S  N

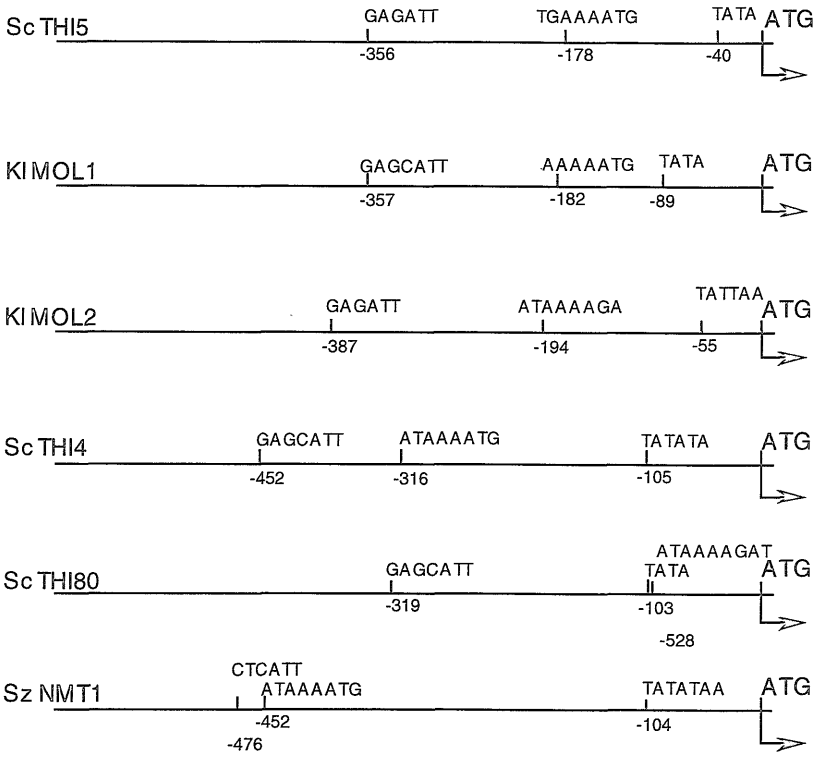
1034 CTACACTAATGAATACTTGTCCTGGCCAGAACCAGAAGAGGTTTCTGATCCTTTGGAAGCTCAAAGATTGATGGCTATTCATCAAGAAAAATGCAGACAGGAAGGTACTTTCAAGAGATT
      Y  T  N  E  Y  L  S  W  P  E  P  E  E  V  S  D  P  L  E  A  Q  R  L  M  A  I  H  Q  E  K  C  R  Q  E  G  T  F  K  R  L

1154 GGCTCTTCCAGCTTAAACGCACTTCGTCACTCCTCGAGCGCAGCCTTCCTCTACGACTTGTGTATGATTTCACATTAGGTTTTTTTTCCTCCCTGATATTTAGATATTTACTATTAAGAT
      A  L  P  A  *

1274 ATTATTTATGAGCCTTTTTATTACAGCTTCTTTTCCCTTGTAATTATTGTACTTGCCTCCAAGTATCGAAAGTCTTGCGTTGCCTCAATTTGCCACTTCAAATACTGTAATCTGCACAATT
1394 TACGTCCGAAATAGATGAGGGATAAAAGCTGTTTTTAAGTACGGCTTATTCAAAAGTATAAAAGTAGAGAGCATAGAATATATAAGGGCATATTTCTTACACAAACGCACCAAAAAAAAA
1514 AAAATCTGATTAATCGAAGGAAATCTGCGCAGACATTGCCGTGAGAAGTGAAGCCTTCTT
```

between -310 to -452 bases from the proposed ATG translation start site in all the respective thiamine regulated genes (figure 7.2). In a promoter deletion of *Sc. THI4*, a deletion -365nt, 5' to the ATG translstion start site results in the dramatic decrease in gene transcription (U. Praekelt, personal communication), therefore, the element involved in the control of *Sc. THI4* by thiamine is 5' to this region. The *K. lactis* homologues to *Sc. THI4* and *THI5* genes (*KMOL1* and *KMOL2* respectively) have been shown to be functional at a slightly reduced level when transferred into *S. cerevisiae* on a yeast episomal plasmid (D. Walsh, personal communication). These observations support the idea that the consensus sequence 5' nGAGCTTn 3' could be involved in the thiamine control mechanism, which is conserved between different yeast species. Another possible element (5' nATAAAATGn 3') was also identified in all the thiamine regulated genes examined, between -100 to -200nt from the translation start site (in the case of *Sc. THI4* at -452nt from the translation start site). The significance of these elements, although present in the deletion region affecting *Sc. THI4* transcription was not determined in this study and their role in thiamine regulation has yet to be identified.

Within the promoter region of *S. pombe nmt1a*, two other elements have been identified, where deletion of TATATAAA sequence 25 bp 5' to the translation start site resulted in a decrease in promoter strength (Basi, Schmid, and Maundrell, 1993) and the promoter element (5' nAAAATCAAn 3'), which has been reported in the *Sc. THI4*, *PHO3* and *THI6* promoter regions (Nosaka *et al.*, 1994). However, this element was not identified within the *THI5* promoter region or the *THI4* promoter region (-595 to -494nt to the translation start site), where a reduction in expression levels is observed, when this region is deleted (U. Praekelt, personal communication). Reporter gene constructs of the*THI5* and *THI12* promoter regions (chrVI and chrXIV, respectively) have been shown to be induced in the absence of thiamine, where both showed a similar level of induction (R. Burrows, personal communication). FASTA comparison of these two promoter regions (upto the transcription start site) shows an 90.6% identity over 676nt (data not shown). The proposed elements were conserved between both these promoter regions. Confirmation that the proposed elements identified are involved in a thiamine mediated control mechanism of the *THI5* gene would require the construction of a series of promoter deletions connected to an appropriate reporter gene (e.g. *E. coli* ß-galactosidase) and the level of transcription determined. Mutation or removal of these sites can then be used to assess the effect this has on transcription levels. Both methods would allow the role of each element to be defined.



Promoter Elements:

Sc THI5	TAT **GAA AAT G**GT
Sc THI80	TT**A TAA AA. G**AT
Kl MOL2	A**CA TAA AA. G**AC
Sc THI4	T**CA TAA AAT G**TC
Kl MOL1	AA**C TAA AAT** TAT
Sc PHO3	AA**C TAA AAT** TTG
Sz nmt1	AT**C TAA AAT** AAT
Sc THI5	C GAC **GAG .AT TGG** CGC
Kl MOL2	T AAT **GAG .AT TGG** ACT
Kl MOL1	C AGT **GAG CAT TC**T AGT
Sc THI4	T TAT **GAG CAT TC**C TC
Sc THI80	A TGG **GAG CAT T**TT TGC
Sz NMT1	A AGC **CTC .AT T**AC AAA
Sc PHO3	A **GAG .AT CGC**

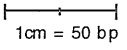


Figure 7.2: Proposed promoter elements in the thiamine regulated genes. Comparison of thiamine regulated genes 5' regions to the transcription start site were compared from yeast species *Saccharomyces*, *Kluyveromyces* and *Schizosaccharomyces*. CLUSTAL V analysis identified two proposed elements. The consensus between these element sequences is shown. Each element showed a conserved location within the promoter region, with their location indicated in the respective promoter region, with figures indicating the number of bases relative to the ATG translational start site. Proposed TATA sequences are also shown. References for the individual genes are as indicated in the text.

The *Sc. THI5* gene encodes a protein, three hundred and forty amino acid residues in length, with a 38.5 kDa calculated molecular weight. The *THI5* peptide showed a 62.0% identity to the thiamine regulated gene, *nmt1a* in *S. pombe* (see Chapter six), whereas the *K. lactis* homologue, *KMOL2* encodes a peptide of three hundred and forty amino acid residues in length, with a 60.0% identity to *nmt1a* (D. Walsh, personal communication). Multiple alignment of the primary amino acid sequences of the different Thi5p homologues by CLUSTAL V, show that the primary structure of these polypeptides is highly conserved (figure 7.3a), with several regions between the individual peptides 100% identical. Another homologue to the *Sc. THI5* has been recently identified in the filamentous fungi, *Aspergillus niger* (Cary and Bhatnagar, 1995).

The hydropathicity profile of the Thi5p (chrVI), provides an indication to the secondary structure of the protein (figure 7.3b). A hydropathicity profile involves the sliding summation of the average hydropathy of each residue along the polypeptide sequence. Where hydrophobic regions found in apolar environments, usually the interior of the protein or membrane spanning domain can then be identified. The hydropathicity profile of the *Sc. THI5* peptide with a sliding average between nine residues using the algorithm of (Kyte and Doolittle, 1982), indicated no membrane spanning domains and was consistent with the profile of a cytoplasmic protein. The *Sc. THI5* peptide showed the characteristic N-terminus of a cytosolic protein, with a slightly hydrophilic region and a overall hydropathicity profile which suggested a globular protein, with a high degree of hydrophilic residues. The major structural motifs detected in the peptide sequence were α helices as determined by the algorithm of (Eisenberg *et al.*, 1984) (data not shown). The regions of homology shown between the *THI5*, *KMOL2* and *nmt1a* proteins, when transcribed to the hydropathicity profile of *Sc. THI5* indicate that the major conserved domains are hydrophobic, combined with the conservation of the primary amino acid sequence, this would indicate that these sites must be functionally important and could indicate the possible location of active sites within the respective proteins (figure 7.3b). This functional conservation can only be confirmed by the restoration of the *THI5* phenotype in a null mutant by a homologue of another species. Mutation of residues within these regions and the effect this has upon the yeast cell, should also be examined as this would indicate the location of active sites within the protein.

Figure 7.3: Comparison of *THI5* polypeptide homologues and hydropathicity profile. **A:** CLUSTAL V alignment of the primary amino acid sequences of the *Sc. THI5* homologues, *KMOL2* (*K. lactis*) and *nmt1* (*S. pombe*). Conserved regions are indicated as shaded boxes, individual regions of conservation are shown as '*' and neutral conservation is indicated as '.'. **B:** Kyte and Doolittle hydropathicity profile of *Sc. THI5* peptide, shaded areas show the major conserved regions (10+ amino acid residues) between the aligned peptides (**A**).

A:

```
SPNMT1A    METMKITPATNWEATPYHLPTFLAQTRGYYERESIEVALLEPTNESDVTALIGSG
KTHI5      MSSDKTTPLLNWQAAPYHVPVLLSMKGYFKEQGIDVALLEPGMPSDVTELIGSG
SCTHI5     METDKITPLLNWQPTPYHIPTFLAQTKGYFKEQGLDMALLEPTNPSDVTELIGSG
           ..||..... ..|||...|.||..  |..|  |.|||.....|...... .....

SPNMT1A    KVDMGLKAMIHTLAAKARGYPVTSFGSLLNEPFTGLITLKGNGI-NDFKDIKGKP
KTHI5      KVDMGLKAMIHTLAAKARGYDVTSIASLLDEPFTGVLYLEGSGITQDFNSLKGKK
SCTHI5     KVDMGLKAMIHTLAAKARGFPVTSVASLLDEPFTGVLYLKGSGITEDFQSLKGKK
           ..................| ... |...|.....|| . .|.. |..| |...|

SPNMT1A    AGYVGEVGKIQLDDLCSKFGLSPSDYTAIRCGMNIAPAIINGEIDGGIGIECMQQ
KTHI5      AGYVGEPGKIQIDELTKHYGMKPTDYEAVRCGMNVAKYIIEGKIDAGVGIECMQQ
SCTHI5     AGYVGEPGKIQIDELTKHYGMKPEDYTAVRCGMNVAKYIIEGKIDAGIGIECMQQ
           ..........|.|.   |.| . .. .|.....|.  ..|. ..|.|.......

SPNMT1A    VELERWCVSQGRPRSDVGMLRIDRLANLGCCCFCTILYIAHDEFIAKHPDKIKAF
KTHI5      VQLEEYLKEKGRDPKDAKMLRIDELACLGCCCFCTILPIANDKFLAENPVKVKKF
SCTHI5     VELEEYLAKQGRPASDAKMLRIDKLACLGCCCFCGVLYICNDEFLKKNPEKVRKF
           .|..     |..   . |..... .. ........|... |. .|  |. .|| .

SPNMT1A    LRAIHSATLDMLKDPVQTYKEYIHFKREMGSELHREGFERCFAYFGHDISNVPRD
KTHI5      LNAIKKATDLVLQDPEQAWKDYVDFKPQLNDPLSYKGFQRCFAYFGSSLYNVHRD
SCTHI5     LKAIKKATDYVLADPVKAWKEYIDFKPQLNNDLSYKGYQRCYAYFGSSLYNVHRD
           .|..  ..  |. .. || .|.||.. ||   .  .||..|....  | .. ..

SPNMT1A    WRKVTNYSKRLGLIPQDFEPNCTNGYLTWELDPDEKDPMGKQEAIAEIQDEIKQK
KTHI5      WRKVTAYGERLDLLPTDFKSNYTNEYLSWEEPTEVDDPLAAQRLIKAHQEACRDQ
SCTHI5     WRKVTGYGKRLALLPPDYVSNYTNEYLSWPEPEEVSDPLEAQRLMAIHQEKCRQE
           .|... .|... .|. .| |. .. ..|.    | ..|  .  |  .|  ||

SPNMT1A    GGVFSGNSLRYVEPANL*
KTHI5      GGFFKRVELK*
SCTHI5     GTF-KRLALP----A*
           .     .
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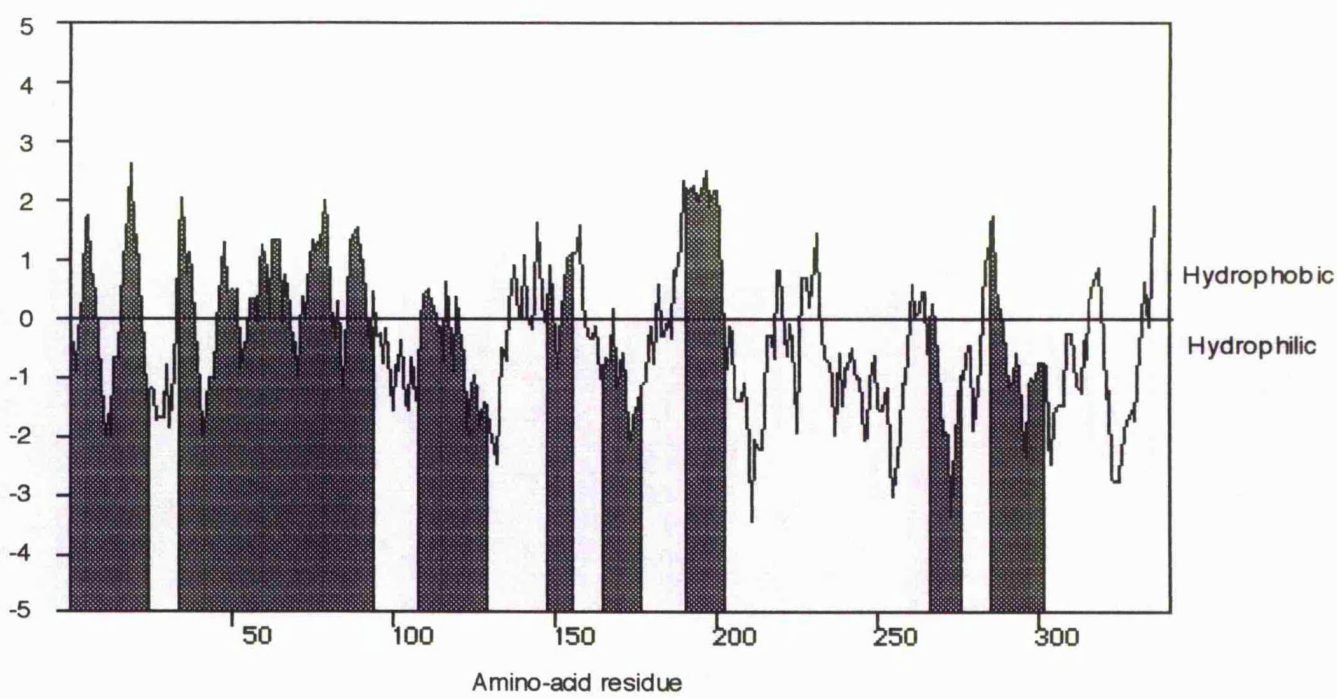
B:



7.3 *THI5* is a thiamine regulated gene encoding a mRNA transcript of 1.4 Kb.

To confirm that the isolated gene, *THI5* was the functional gene and that transcription was regulated by extracellular thiamine levels, DNA sequence representing the unique 3' untranslated region of the mRNA was hybridized to total cell RNA isolated from exponential and stationary phase yeast cells grown on Whickerham's minimal media supplemented with thiamine-HCl (+B1) to a concentration of 2.0μM. The 3' region of RH4/11 was released on a *BsgI*/*Sap*I restriction fragment (1689 to 1896nt) and used as 'probe' DNA.

S. cerevisiae cells were grown on Whickerham's minimal media supplemented with 2.0%(w/v) glucose, without thiamine, overnight at 28°C. This culture was used to inoculate two cultures of minimal media; one supplemented with extra-cellular thiamine to 2.0μM, the other as a control culture containing no thiamine. After a cell density of 5.0x 10^6 cells ml^{-1} was reached, cell samples were taken every two hours and total cell RNA extracted.

'Northern' hybridization analysis with the *ESP30* cDNA as probe, showed that *THI5* expression in cells grown on thiamine supplemented media (+B1) was repressed. Whereas in cells grown on the non-supplemented medium (-B1), expression was constitutive (data not shown). The 3' untranslated region of the *THI5* gene, showed the same hybridization pattern as that of the *ESP30* cDNA (figure 7.4). The transcript detected was sized by comparison to a known size marker to 1.40Kb (marked by a black triangle, see figure 7.4).

7.4 Transcript detection from *THI5* and *THI2*

As the chromosome VI locus has been shown to be identical to the *ESP30* cDNA sequence and thus must have been transcribed, the question arises is the chromosome XIV locus also transcribed?. From the published DNA sequence, the chromosome XIV locus is capable of encoding a predicted protein of three hundred and forty amino acid residues in length. As the chromosome VI locus also encodes an identical polypeptide, therefore, if both loci are transcribed, one signal would be detected by 'Northern' hybridization as both loci have the potential to produce transcripts that would be of the same size. Confirmation that the chromosome XIV locus is transcribed was determined by a combined 'dot-blot' 'Southern' and 'Northern' hybridization of the 3' region of the individual chromosome VI and XIV ORF's, to total cell RNA from yeast cells grown on Whickerham's minimal media + and - thiamine (see section 7.3). The 3' region to each locus was isolated by PCR

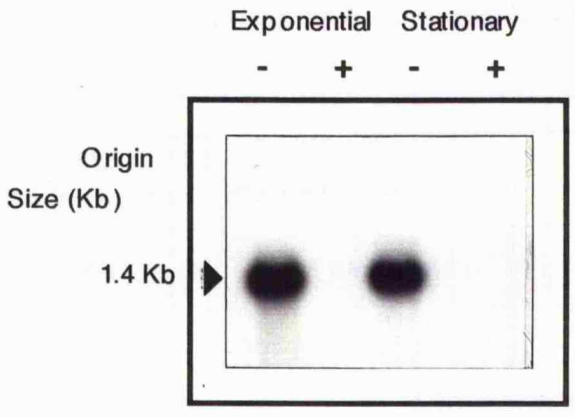


Figure 7.4: 'Northern' hybridization of mRNA isolated from yeast cells grown on +/- thiamine supplemented Wickerham's minimal media. mRNA was isolated from exponential and stationary phase S288Ca yeast cells grown on (+) or (-) thiamine supplemented media. Probe DNA is as indicated in text, with hybridization in Church-Gilbert buffer at 65°C, followed by two, high stringency washes at 65°C (3x SSC, 0.1%(w/v) SDS). The size of the transcript detected was determined by comparison of migration distance with a known standard (0.16Kb - 1.77Kb RNA ladder BRL Ltd).

amplification from the plasmid clones pRH4 and pRH3 using the specific oligonucleotide primer combinations, TP1/TP2 (chrVI) and TP1/TP3 (chrXIV), respectively (figure 7.5a). Oligonucleotide primer TP1 was specific to the conserved region 3' to the transcription termination signal of the *THI5* ORF in both chromosome VI and XIV loci and within the region of homology to the *ESP30* cDNA (figure 7.5a). Primers TP2(VI) and TP3(XIV) were specific to the individual chromosome loci and corresponded to sequence 3' to the region of homology to the *ESP30* cDNA. The length of the PCR product was chosen to be ≈100nt, thus ensuring that this region would span the sized transcript product of the chromosome VI locus and the potential transcript of the chromosome XIV locus.

The DNA polymerase chain reaction (PCR) was performed in a total reaction volume of 20µl (see Materials and Methods, section 9.8.13) and due to the small size of the expected PCR product, there was no extension period required, with the following cycles used; 1 cycle 95°C 280 seconds, followed by 25 cycles 95°C 30 seconds, 55°C 30 seconds. A 'dot-blot' hybridization filter was prepared (see Materials and Methods, section 9.8.10), with 2µg total cell RNA isolated from S288Ca cells grown on minimal media + or - thiamine (+B1 and -B1, respectively) (see section 7.3) and ≈0.5µg PCR product TP1/TP2(VI) and TP1/TP3(XIV) as determined by agarose gel electrophoresis against known amounts of restriction endonuclease cut plasmid DNA. The remaining PCR product was labelled by random hexamer priming (see Materials and Methods, section 9.8.11) and were hybridized at 65°C in Church-Gilbert buffer. After this hybridization period, the filter was washed twice in 3xSSC/0.1%(w/v) SDS at 65°C (see Materials and Methods, section 9.8.8).

Both TP1/TP2(VI) and TP1/TP3(XIV) detected a transcript in total cell RNA isolated from yeast cells grown on Whickerham's minimal media -thiamine (-B1), with no transcript detected in cells grown on minimal media +2.0µM thiamine (+B1) (figure 7.5b). However, the PCR products showed a cross hybridization to each other and therefore could be detecting the same transcript (figure 7.5b). When hybridized to the *ESP30* cDNA, a transcript was detected in minimal media -thiamine (-B1), but not in +2.0µM thiamine (+B1). The *ESP30* cDNA gave an higher intensity hybridization signal to the TP1/TP2(VI) PCR product, then to the TP1/TP3(XIV) PCR product (figure 7.5b). This would be expected as the *ESP30* cDNA is identical to the TP1/TP2(VI) product, however, the *ESP30* cDNA does show a reduced hybridization signal to the TP1/TP3(XIV) PCR product. Therefore, the transcript detected by the TP1/TP3(XIV) product could be a result of the transcription of the chromosome VI locus and not the transcription of the chromosome XIV locus.

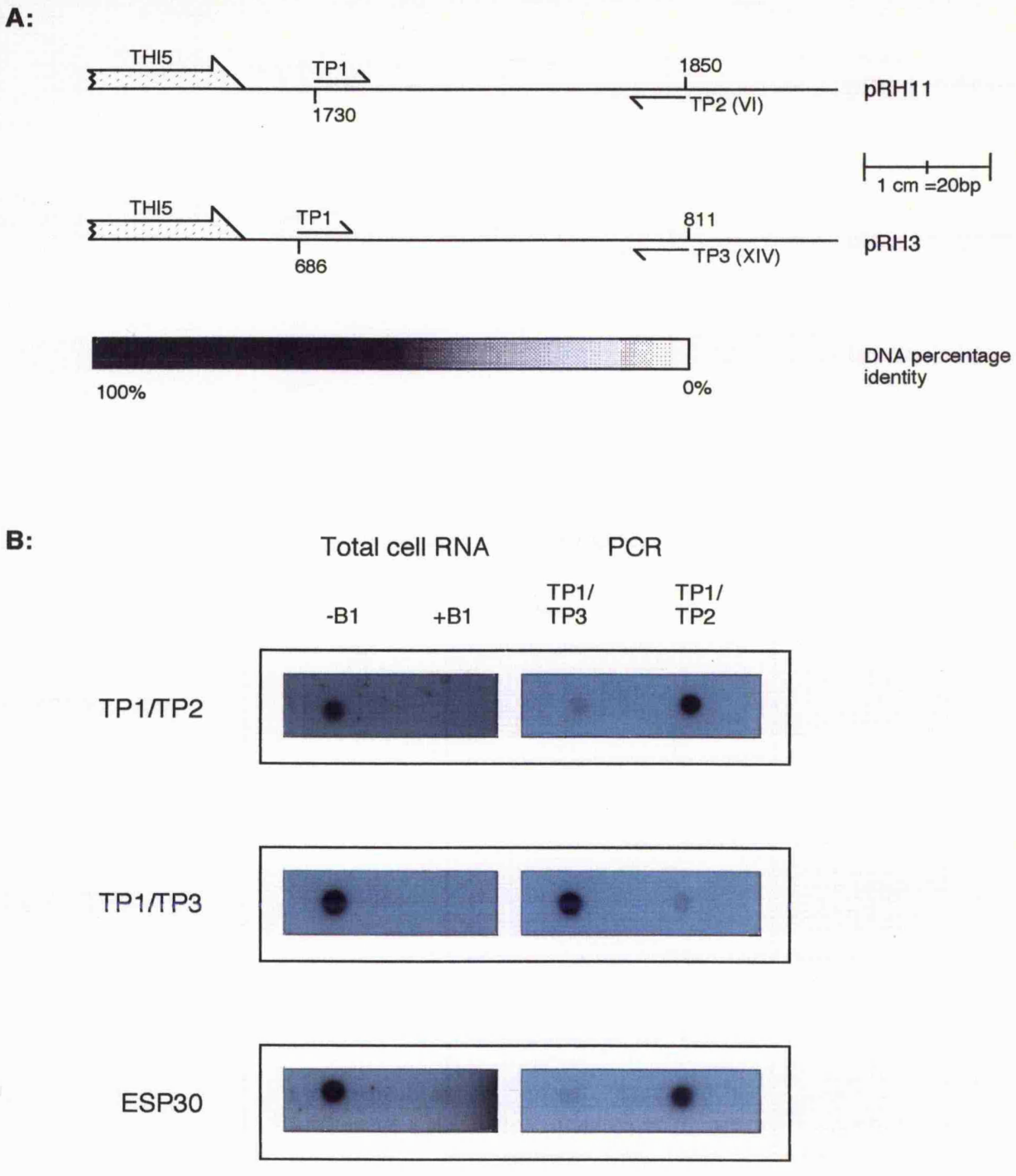


Figure 7.5: Transcript detection by dot-blot hybridization. **A:** The 3' region to the transcription termination codon of the THI5 ORF was isolated from plasmid clones pRH3 and pRH4 specific to chromosome XIV and VI loci, respectively. Primer TP1 was common to both loci, with the remaining primers specific to the individual chromosome loci, as determined by sequence comparison. TP2 (VI) and TP3 (XIV) were designed to be ≈100nt 3' to the TP1 primer and within the transcript size. Comparison of these THI5 3' untranslated regions shows that towards the TP2 and TP3 the level of DNA identity decreases. **B:** 'Northern' and 'Southern' hybridization using PCR generated chromsome specific regions as hybridization probes to 2.0μg total cell RNA isolated from S288Ca yeast cells grown on Whickerham's minimal media +/- thiamine and unlabelled PCR products.

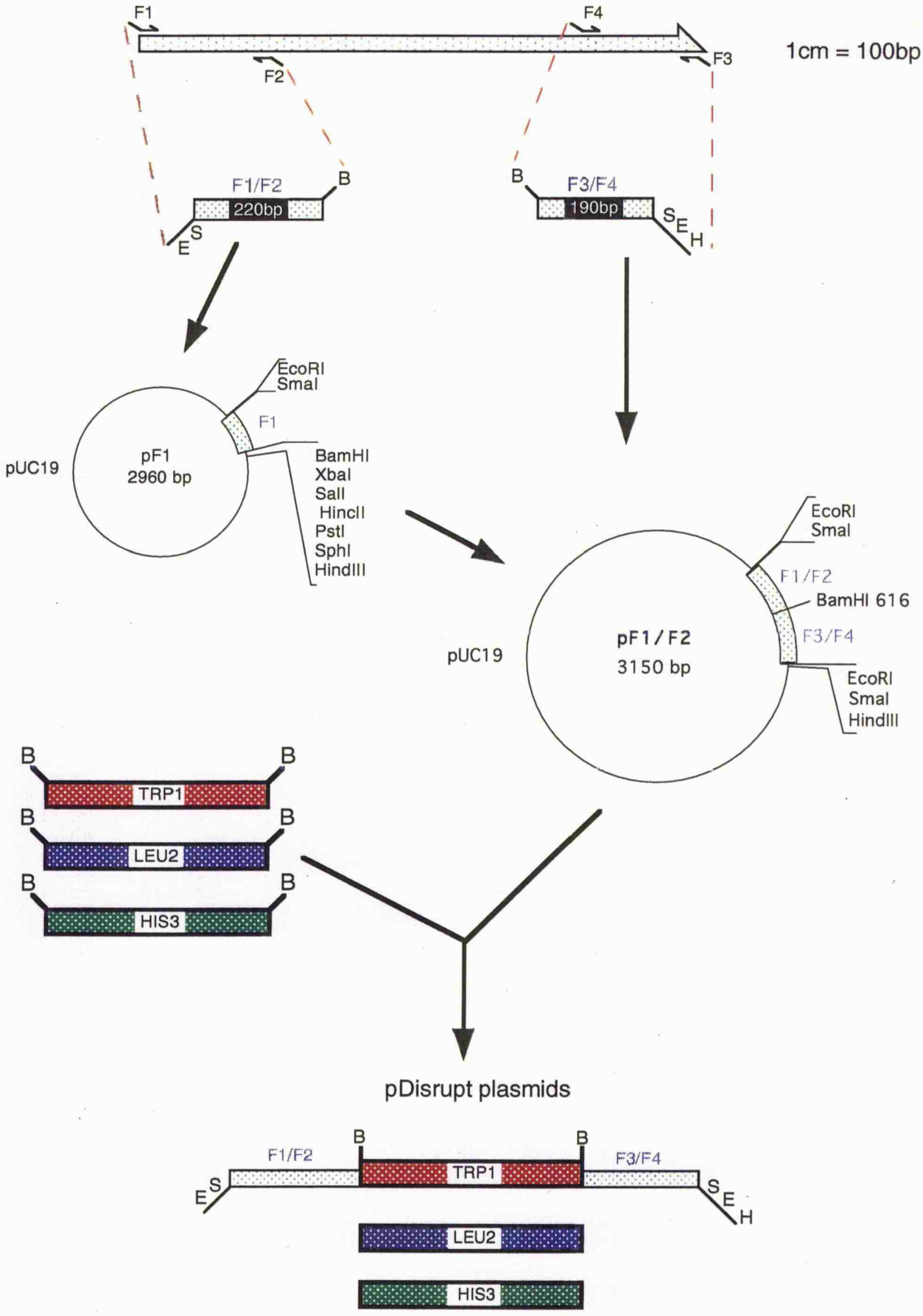
7.5 Gene disruption of *THI5* and *THI12*

As the *THI5* gene family members could not be distinguished by transcription detection, the next stage in their analysis was the creation of a series of gene disruptions within the different chromosome loci and examination of the resultant phenotypes. Disruption of the different*THI5* loci would determine which were functional and also allow the role of *THI5* protein in thiamine biosynthesis to be identified. A null mutant in each chromosome locus was created by *in vivo* homologous recombination with a disrupted open reading frame derived from the chromosome VI locus, constructed *in vitro*. Growth of these null mutants was examined on media supplemented with the reported precursors involved in thiamine biosynthesis (Young, 1986). The design of the disruption cartridge for the production of the null mutant is described below (figure 7.6).

As the coding sequence of the chromosome VI and XIV loci is 99.3% identical (at the DNA level), then a disruption cartridge formed from the chromosome VI ORF could be used to disrupt both the chromosome VI and the other loci. Flanking sections of the 5' and 3' regions of the *THI5* ORF from the plasmid clone pRH11 were formed by PCR amplification. The oligonucleotide primers used (appendix one), contained engineered restriction endonuclease sites to facilitate the cloning of these fragments into the plasmid vector, pUC19. The initial plasmid constructed, contained the 5' region of the *THI5* ORF corresponding to +1 to +232nt (amino acid residue 1 to residue 77) and was termed pF1 (figure 7.6), where engineered restriction sites *Eco*RI and *Bam*HI on oligonucleotide primers F1 and F2, respectively enabled this 232nt DNA fragment to be cloned into pUC19. The second DNA fragment corresponding to the 3' region of the *THI5* ORF (280 to 334 amino acid residues) was isolated as a 163nt PCR product. Engineered restriction endonuclease sites, *Bam*HI and *Hin*dIII on oligonucleotide primers F3 and F4, respectively were used to clone this fragment into plasmid pF1, forming the plasmid pF1/F2, which has a unique *Bam*HI restriction endonuclease site, flanked by the 5' and 3' portions of the *THI5* ORF. This arrangement allows for the integration of a number of yeast biosynthetic genes into the unique *Bam*HI site, with engineered restriction endonuclease sites (*Eco*RI and *Sma*I), flanking this construction, which allow for the easy excision of the created gene disruption cassette (figure 7.6).

Three distinct disruption cartridges were constructed to disrupt the three different chromosome loci. Yeast biosynthetic genes were used as selective markers in the disruption strains and were obtained from the YDp series of plasmids which contain several yeast biosynthetic genes, flanked by a unique *Bam*HI restriction endonuclease

Figure 7.6: pDisrupt construction strategy. *THI5* ORF was used in the construction of a series of disruption cartridges. The 5' region of the *THI5* ORF, corresponding to the first 77 amino acid residues was isolated by PCR with modified oligonucleotide primers F1 (containing an *Eco*RI and *Sma*I restriction endonuclease sites) and F2 (containing a *Bam*HI restriction endonuclease site) from plasmid pRH11. The PCR product was digested with *Eco*RI and *Bam*HI, prior to cloning into pUC19, to form the plasmid pF1. The PCR product of the 3' region of *THI5* ORF (corresponding to amino acid residues 288 to 334), was cloned into plasmid pF1 on *Bam*HI and *Hin*dIII restriction endonuclease sites, to form the plasmid pF1/F2. This was used to clone into the now unique *Bam*HI site, yeast biosynthetic genes from the YDp plasmid series, to form the disruption cassettes pDisrupt-L, -W and -H.


F1
F4
F2
F3
1cm = 100bp
F1/F2
220bp
B
S
E
B
F3/F4
190bp
S
E
H
EcoRI
SmaI
F1
pUC19
pF1
2960 bp
BamHI
XbaI
SalI
HincII
PstI
SphI
HindIII
EcoRI
SmaI
F1/F2
BamHI 616
F3/F4
pUC19
pF1/F2
3150 bp
EcoRI
SmaI
HindIII
B
B
TRP1
LEU2
HIS3
pDisrupt plasmids
F1/F2
B
B
F3/F4
TRP1
S
E
H
LEU2
HIS3

site (Berben *et al.*, 1991). The yeast biosynthetic genes, *LEU2*, *TRP1* and *HIS3* were released from the YDp plasmid series on a unique *Bam*HI restriction endonuclease site and cloned into the plasmid, pF1/F2 to form the disruption cassettes termed pDisrupt-L, pDisrupt-W and pDisrupt-H, respectively (figure 7.6).

A 'one-step' gene disruption protocol as described by (Rothstein, 1983) was used to form the *THI5* null mutants. The initial stage in the construction of these mutants was the disruption of each individual locus. The first locus disrupted was chromosome VI, where 20.0µg pDisrupt-L plasmid DNA was digested with *Sma*I to release the disruption cartridge as a 2.0Kb linear DNA fragment. The haploid yeast strain YPH500 (Sikorski and Hieter, 1989), isogenic to S288Ca was transformed with this linear DNA disruption fragment. Genomic DNA was isolated, from transformed yeast cells which showed a LEU+ phenotype, when grown under selective pressure on SD medium -LEU, +thiamine (2.0µM), +2%(w/v) glucose. Mutants in the *THI5* locus were identified by 'Southern' hybridization of *Xho*I digested genomic DNA, to the *THI5* ORF. Mutants in the chromosome VI locus were identified by a predicted 1.0Kb shift in the hybridization signal, when compared to the 'wild-type' (non-mutated parental strain) hybridization signal at 3.8Kb. This shift in DNA fragment size indicated that integration of the disruption cartridge containing the *LEU2* gene had occurred (figure 7.7).

The next stage in the evaluation of the *THI5* homologue family was to disrupt *THI12*, using the disruption cassette pDisrupt-W. The same procedure was used as with the pDisrupt-L construct. The pDisrupt-W disruption cassette was released as a 1.2Kb *Eco*RI restriction endonuclease generated DNA fragment. The haploid yeast strain YPH499 (Sikorski and Hieter, 1989) was transformed with this linear DNA fragment and a null mutant in the *THI12* locus was identified by genomic DNA 'Southern' hybridization to the *THI5* ORF. From sequence data (van Dyck *et al.*, 1995), a predicted 10.16Kb size *Xho*I DNA fragment contains the *THI2* locus. A mutant in the chromosome XIV locus was identified by a shift in size (0.2Kb) of the hybridization signal upon the integration of the *TRP1* disruption cartridge (figure 7.7). As the hybridization signal of the *THI12* (chromosome XIV locus) was in a region above the linear migration of DNA fragments, confirmation of the disruption was by 'Southern' hybridization with the biosynthetic gene marker, *TRP1*, where two hybridization signals were detected, which confirmed the disruption of the locus (data not shown). Allocation of chromosome location in both the VI and XIV loci was by estimation of the size of the DNA fragment generated and comparison to sequence data (chapter six and appendix II, III). In the 'wild-type' (non-mutated parent strain), a *Xho*I restriction endonuclease generated chromosomal DNA fragment of 3.8Kb

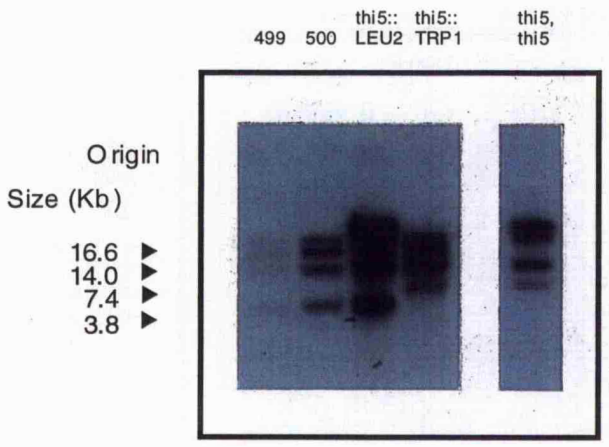


Figure 7.7: 'Southern' hybridization of genomic DNA from Δ*THI5* disruption strains. *Xho*I restriction endonuclease digestion of genomic DNA isolated from the parental strains; YPH499 (499) and YPH500 (500) (see text) and the disruption strains *thi5*::TRP1+ and *thi5*::LEU2+, hybridized with the *THI5* (chrVI) open reading frame PCR product as a probe fragment under high stringency conditions. A shift is observed in the hybridization signal of the disrupted strains indicating integration of the disruption cassette into the *THI5* (chr VI) and *THI5* (chrXIV) loci. The size of each hybrization signal is indicated by a black triangle (see text for details).

contains the *THI5* gene located on chromosome VI, whereas a 10.16Kb *Xho*I fragment, corresponds to the chromosome XIV locus. Two other hybridization signals were detected at 7.4Kb and 14.0Kb, when hybridized to a DNA fragment corresponding to the complete *THI5* ORF; the 14.0Kb hybridization signal was due to the chromosome X locus (see chapter eight). The remaining hybridization signal, appears to be a result of non-specific hybridization, as only three copies of the *THI5* gene were indicated to be located on three distinct chromosomes in *S. cerevisiae* (chapter six). This non-specific hybridization could be a result of the hybridization of the 3' region of *THI5* to the open reading frame 'YCR107w', as this region shows a 70% identity over 110bp to the 'YCR107w' gene (data not shown). Integration of the disruption cassettes into the respective loci was confirmed by 'Southern' hybridization with the corresponding biosynthetic marker genes (data not shown).

As the yeast strains used in the construction of the above single *THI5* disruptions were α and **a** mating types, respectively, it was possible to mate these two single mutant strains to form a double mutant, in both the chromosome VI and XIV loci. The single mutant strains, YPH499 Mata, *thi12*::TRP1+ and YPH500 Matα, *thi5*::LEU2+ were mated, by 'drop overlay' (Spencer and Spencer, 1990) of an overnight YEPD broth culture of each strain, onto the selective agar medium, SD -L,-W. From this spot mating, single diploid colonies were isolated, by streak plate culture on the selective medium SD -L,-W, +thiamine (2.0μM) at 28°C for approximately five days. Single diploid colonies were then grown on pre-sporulation medium, prior to sporulation by streaking onto sporulation agar medium (see Materials and Methods, section 9.3.2). After sporulation, spores from individual tetrads were then dissection onto YEPD agar medium and grown at 28°C for three days. The resultant haploid colonies, when replica plated on the selective media, SD -LEU or -TRP, segregated 2:2 with respect to the biosynthetic marker genes, integrated into the different *THI5* homologue loci (data not shown). A single haploid colony was isolated that contained both biosynthetic markers and hence was disrupted in both *THI5* and *THI12*; this yeast strain was termed *thi5*::LEU2+, *thi12*::TRP1+ (*thi5*, *thi12*). 'Southern' hybridization of the *THI5* ORF to genomic DNA isolated from this double mutant, confirmed that both loci were disrupted (figure 7.7). In the diploid parent strain to *thi5*, *thi12*, six hybridization signals were detected when genomic DNA was hybridized to the *THI5* ORF (data not shown), which indicates that the diploid strain contains both non-mutagenised and mutagenised loci.

Yeast mutant strains, YPH499 *Mata*, *thi12*::TRP1+, YPH500 *Matα*, *thi5*::LEU2+, were grown at 28°C for three days under non-selective pressure on SD medium (supplemented with thiamine (2.0μM) and the amino acids necessary for growth (see

Materials and Methods, section 9.3.3)). The identified null mutants YPH499 *thi12*::TRP1+, YPH500 *thi5*::LEU2+ and *thi5,thi12* were used to determine the effect of the *THI5* disruption on growth in yeast cells on thiamine and its precursors. Growth of these disruption strains and control strains YPH500, YPH499, PMY3 (*Mata, ura3-52*) and PMY3::*thi4* were examined on Whickerham's minimal media agar, supplemented with 2%(w/v) glucose and the known precursors in the thiamine biosynthetic pathway; + hydroxymethyl-pyrimidine (2.0μM), + hydroxyethylthiazole (2.0μM), -thiamineHCl (-B1) and +2.0μM thiamineHCl (+B1). Three successive periods of incubation (72 hours each) were used to ensure any residual growth was not a result of contaminating thiamine, which may have accumulated during initial growth on SD medium supplemented with thiamine.

Both, single null mutant strains, *thi12*::TRP1+ and *thi5*::LEU2+ did not exhibit a thiamine auxotrophic phenotype, when grown on Whickerham's minimal media minus thiamine, whereas the single mutant *thi4*::URA3+ did (figure 7.8). Growth was observed with both the non-mutated ('wild-type') parental strains and null mutant strains on Whickerham's minimal agar medium supplemented with extracellular thiamine (figure 7.8). This pattern of growth indicated that the *thi4* mutant is auxotrophic for extracellular thiamine and *THI4* is essential for normal growth. The *thi4* mutant did not grow in the presence of the pyrimidine precursor, however, growth was observed on medium supplemented with the thiazole precursor (data not shown). This pattern of growth is consistent with the phenotype previously reported, where *THI4* is reported to be involved in the thiazole precursor pathway (Praekelt and Meacock, 1992; Praekelt *et al.*, 1994).

In both single disruption mutants, a thiamine auxotrophic phenotype was not evident. This would indicate that the remaining gene(s) are active, and can functionally complement each other. Therefore, the phenotype of the double mutant, *thi5, thi12* was examined and found also not to be thiamine auxotrophic (figure 7.8), which would indicate that the third gene on chromosome X is active. Both the single *thi5* and *thi12* mutants and the double mutant, *thi5,thi12* showed a normal level of growth on the thiazole and pyrimidine precursor (data not shown). These observations suggest that each of the *THI5* homologues encodes a functional protein that is capable of complementing the function of the others. However, this can only be confirmed by the disruption and examination of the cell phenotype, in a mutant disrupted in all three of the *THI5* homologues.

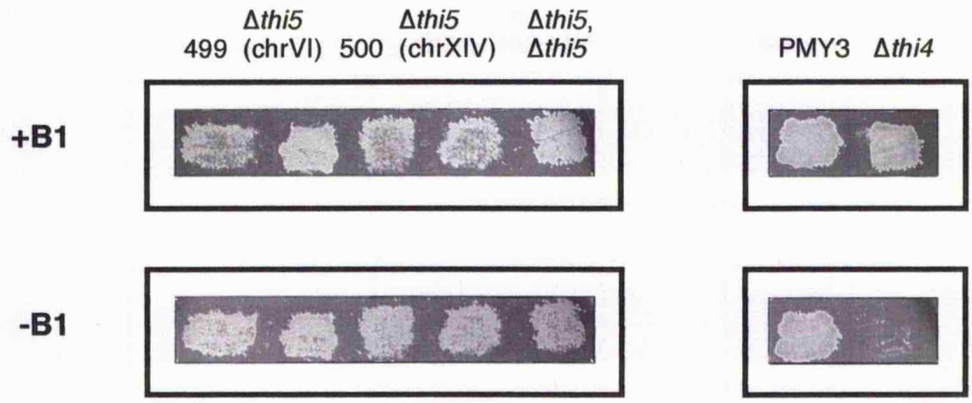


Figure 7.8: Phenotype of Δ*THI5* disruption mutants. Disruption mutants of *THI5* (chrVI) and (chrXIV) were formed by 'one-step' gene disruption with linear disruption cassettes, pDisrupt (see text for details). Single null mutants in *THI5* loci; chrVI (Δ*thi5*(chrVI)) and chrXIV (Δ*thi5*(chrXIV)) and a double mutant (Δ*thi5*,Δ*thi5*) were grown under + (**+B1**) and - (**-B1**) thiamine selective conditions on Whickerham's minimal medium supplemented with all necessary amino-acids. The following parent strains, YPH499 (499), YPH500 (500), PMY3 and the known thiamine auxotrophic mutant, PMY3::*thi4* (Δ*thi4*) were used as control strains. Three successive replications and periods of growth (at 28°C) were used to ensure the phenotype observed was stable and characteristic of the cell genotype.

7.6 Conclusions

THI5 is a thiamine regulated gene, homologous to *S. pombe nmt1a*, which is involved in the pyrimidine biosynthesis pathway leading to thiamine. Both the *THI5* and *THI12* (chromosome VI and XIV loci, respectively) encode for a protein of three hundred and forty amino acid residues, with a calculated molecular weight of 38.5 kDa. Multiple copies of this gene appear to be present within the *Saccharomyces* genome, with active genes localised to chromosome VI, X and XIV.

Two possible upstream elements were identified in the 5' region to the translational start site, where these elements appear to be common within various thiamine regulated genes of different yeast species. The observation that the promoter regions of the *K. lactis* homologues to both *Sc. THI4* and *Sc. THI5* are functional in *S. cerevisiae* support the idea of a universal and conserved mechanism of control via external thiamine concentration. The identified conserved elements could be part of this mechanism, however, this can only be confirmed by promoter deletion and mutagenesis of these elements. Reported elements found in the *S. nmt1a* and *nmt2* genes (Manetti *et al.*, 1994) were not identified in the comparison. Both *nmt1a* and *nmt2* in *S. pombe* have been reported to be coordinately expressed (Manetti *et al.*, 1994). Both *THI4* and *THI5* in *S. cerevisiae* and their respective *K. lactis* homologues also exhibit a coordinate pattern of expression (chapter two). This would suggest that the gene products encoded by these genes are required at the same time in the biosynthesis pathways and again support the idea of a general control mechanism responding to thiamine concentration.

Disruption of the *THI5* and *THI12* suggested that *THI11* on chromosome X is also functional. The conserved nature of both chromosome VI and XIV loci (chapter six) suggests that a selection pressure has been maintained, and would account for the observed phenotype of each mutant. As single and double mutants do not exhibit a thiamine auxotrophic phenotype, this would indicate that all three loci are capable of mutual compensation. In contrast, the *THI5* homologue in the *Schizosaccharomyces pombe* genome, *nmt1a* is reported to be present as a single copy, which when disrupted results in a thiamine auxotrophic phenotype (Maundrell, 1990). The question then arises, why does the *S. cerevisiae* cell require three active copies of the same gene?. One possible explanation for this, is the observed activity of the *Sc. THI4* and *Sc. THI5* / *Sc. THI12* promoters, where the fusion of the *Sc. THI4* and *THI5* promoter regions to reporter gene constructs indicates that the *THI4* promoter is induced to a level double that of the *THI5* and *THI12* promoters (R. Burrows,

personal communication). Therefore, *in vivo*, *THI4* could be expressed to a higher level than that of *THI5*, which would then account for multiple copies of the *THI5* gene which would be necessary to maintain the balance of the precursor moieties within the thiamine biosynthetic pathway. However, this still does not answer the question of why three copies of the *THI5* gene are present within the yeast genome and why their continual selection is necessary (as indicated by their conserved nature). This continual selection and hence conserved nature of the *THI5* gene is mirrored in the preservation of the primary amino acid sequence between various fungal *THI5* homologues. Where the conservation of structure observed could indicate that the *THI5* gene product is essential in these species and also provides an indication of possible active sites within the Thi5p, however, this can only be determined by experimental analysis, employing a mutagenesis strategy.

In conclusion, multiple copies of *THI5* exist in the yeast genome, where both *THI5* and *THI2* are expressed. Both the *THI5* and *THI12* have been shown to be thiamine regulated, with both encoding a potential protein ≈38 kDa in size. Both loci contain a functional gene and appear to complement each other, with a proposed function for both the *THI5* and *THI12* gene product in the biosynthetic pathway leading to the thiamine precursor, hydroxymethyl pyrimidine.

Chapter 8: General discussion

In this thesis, the apparent coincident regulation of a set of six genes, initially identified as a molasses grown yeast batch culture transitions through respiratory into stationary phase, was examined. Induction of these genes corresponded with the depletion of external hexose source(s) and the subsequent utilization of respiratory substrate(s). The studies undertaken in this thesis, showed that the pattern of expression was a result of the complex nature of the molasses substrate, and the subsequent response by these genes to different control mechanisms. From the initial coordinate pattern of regulation exhibited in a molasses grown culture, further studies involving growth under defined genetic backgrounds and different culture conditions, allowed the genes to be defined into three distinct groups based upon their pattern of regulation. *ESP65* (*HXT4*) and *HXK1* were grouped as controlled via a catabolite de-repression mechanism; *HSP12* and *HSP26* were identified as previously reported small heat shock proteins, where their induction was found to be a response to the physiological status of the cell detected via a mechanism distinct from the carbon catabolite repression pathway; *THI5* and *THI4* expression was specific to blackstrap molasses cultured cells, where a transient induction of both genes was as a result of the depletion of a physiological factor within the cell/environment, not related to the available carbon source.

Both *HXK1* and *ESP65* (*HXT4*) were previously identified, as hexokinase A and a hexose transport gene, respectively (Bisson *et al.*, 1993; Kopetzki *et al.*, 1985; Prior *et al.*, 1993). From growth studies in non-catabolite repressing growth media and glucose-grown cultures of strains with mutations in the glucose repression pathways (*tup1/ cyc8 /snf1*), both genes were found to respond to a carbon catabolite regulation. The observed pattern of regulation for both genes, is consistent with the function they encode, in that they are involved in hexose metabolism and must therefore respond to external hexose concentration. *HXK1* expression was shown to be regulated via the *TUP1/CYC8*, *SNF1* pathway, with a pattern of induction as reported by Muratsubaki and Katsume (1979). *ESP65* (*HXT4*) was identified as a member of a multigene 'low affinity' hexose transporter family. As the results obtained within this study have shown, *ESP65* is controlled by a glucose repression mechanism, which is consistent with that described for a 'high affinity' hexose transport gene. Therefore, the constitutive nature reported for *HXT4 (LGT1)* and hence the reported affinity of the transporter must be queried. Both *HXK1* and *ESP65* were not further characterized within this thesis as both had been previously described and reported (Muratsubaki and Katsume, 1979, and Prior *et al.*, 1993, respectively).

The second group of genes identified in this study were the previously reported heat shock genes *HSP26* (Petko and Lindquist, 1986) and *HSP12* (Praekelt and Meacock, 1990). In response to adverse conditions, as detected by the yeast cells physiological condition, 'stress' genes are induced in order to preserve cell integrity. One such 'stress' control mechanism, is mediated via intracellular cAMP, which regulates the activity of a cAMP dependent protein kinase. The transition from rapid fermentative growth through respiratory growth into stationary phase is registered as a stress situation, due to a fall in intracellular cAMP. Expression of *HSP12*, and to a lesser degree *HSP26*, responded to the physiological status of the cell as detected via a cAMP-dependent protein cascade mechanism, with *HSP12* also appearing to be regulated via part of the *SNF1* pathway. The change in the environmental status of the cell on entry into the diauxic lag/stationary phase, which results in the high level of induction of these heat shock genes, would suggest a role in cell survival. From the level of transcription observed it would appear that the cell requires these heat shock proteins, however, disruption of either the *HSP12* or *HSP26* gene does not effect cell viability and the double mutant, *hsp12*, *hsp26* is still viable (U. Praekelt, personal communication). This suggests that a further gene or series of genes must be able to complement the function encoded by these two small heat shock proteins.

The pattern of expression shown by both *HSP12* and *HSP26* is very similar, where their induction appeared to be in a response to a change in the physiological status of the cell as detected by a mechanism distinct from the *TUP1/CYC8* glucose catabolite repression pathway. However, both genes did respond to the physiological status of the cell as detected via a cAMP-dependent protein cascade mechanism, which is consistent with the reported synthesis of high molecular weight heat shock proteins, which respond to a decrease in cAPK, i.e. *HSP70* (*SSA3*). Recent reports have indicated that the response to a variety of stress stimuli by a number of known stress genes i.e. *SSA3* in response to diauxic shift/ carbon starvation, *HSP12* to osmotic stress (Varela *et al.*, 1995) and *CTT1* to oxidative stress (Schuller *et al.*, 1994) is mediated through a stress responsive element (STRE). Transcription factors, Msn2p and Msn4p, recognize these STRE's and presumably become phosphorylated by a range of protein kinases which respond to different stresses or metabolic perturbations e.g. the *HOG1* MAPK in response to osmotic shock (Schuller *et al.*, 1994). A variety of stimuli are known to induce the synthesis of the small heat shock proteins, however, the precise physiological signals responsible for their induction remains vague. Further areas of investigation would focus on the localisation within the cell and function of the *HSP12* protein. Also the regulatory pathway which effects these two genes should be examined, as two independent mechanisms appear to be acting upon their expression.

The third class of genes, were found to respond to depletion of an essential metabolite from the cell and its environment. Both, *THI4* and *THI5*, showed a coordinate pattern of induction in response to the depletion of the essential vitamin, thiamine. Previous studies (Praekelt *et al.,* 1992), have shown that *THI4* is involved in the biosynthetic thiazole precursor pathway to the thiamine moiety, whereas a role for *THI5* in the pyrimidine precursor pathway can be inferred from its homology to the *S. pombe* gene *nmt1a* (Maundrell, 1990). Depletion of thiamine, appears to lead to the induction of this biosynthetic pathway(s), where control is exerted by the end-product of the pathway i.e. thiamine. Therefore, the regulation of both *THI4* and *THI5* must contribute to the control of flux through this biosynthetic pathway, which is apparent in the pattern of regulation observed. Comparison of the *THI4* and *THI5* promoter regions identified two elements, with conserved position and structure (see chapter seven). Whether these element(s) of the *THI5* and *THI4* promoters are involved in thiamine regulation remains to be confirmed.

The patterns of gene activity observed, reflect the complex nature of the growth medium, molasses. Undoubtedly, other genes also respond to these controls e.g. *SSA3* is also induced by a decline in cAMP levels following glucose starvation and entry into stationary phase, and also many genes are reported to be controlled through *CYC8/TUP1* (see review for examples (Ronne, 1995). However, as the initial six genes were isolated by a differential screen (Praekelt and Meacock, 1990), we can conclude that in molasses grown yeast cells, no homologues are expressed constitutively, or at other stages within the growth cycle (as supported by 'Northern' hybridization data, unpublished data).

Of the initial six genes identified, the thiamine regulated genes *THI4* and *THI5* were further characterized in this study. Although previous analysis had shown that *THI4* is present only as a single copy within the yeast genome, this study has shown that the pyrimidine precursor biosynthetic gene, *THI5* is present in multiple copies. Four copies were identified by 'Southern' hybridization and localised to specific chromosomes. These copies have been identified on chromosomes VI (YFL058w), X (YJR156c), and XIV (YNL332w). During the writing of this discussion, a fourth copy of the same gene (termed,*THI13* -YDL244w) was identified on chromosomes IV by Alt-Moerbe, Schneider and Moro (EMBL accession No. Z74292). All appear to be capable of encoding a full length product, of 340 amino acid residues.

Of the three *THI5* homologues identified in this study, two were characterized; one functional gene was found to be located on chromosome VI (*THI5*), with a second on chromosome XIV (*THI12*); the third gene locus was not characterised at the molecular level, but was identified by homology searches to the published yeast DNA sequence on chromosome X (*THI11*). The gene promoter sequence from both *THI5* and *THI12*, is functional on plasmids (R. Burrows, personal communication), indicating that both genes encode a functional protein. However, a thiamine prototrophic phenotype was observed in both single *thi5* and *thi12* mutants and in the *thi5*, *thi12* double mutant. This indicated that *THI11* ([EMBL accession no. Z49655], located within the right telomere region (728,268 - 729,287nt) on chromosome X) or the recently discovered *THI13* (EMBL accession no. Z74292) are also functional.

From published DNA sequences, comparison of the *THI5*, *THI11*, *THI12* and *THI13* promoter and coding sequences, indicated a highly conserved sequence structure; where each primary peptide sequence shows an ≈99% identity to the other (figure 8.1). Thus, the '*THI5*' family appears to have been replicated between four different chromosomes and that each member has remained intact. In all four regions, DNA sequence diverges at around +700nt 5' and +100nt 3' of the coding sequence. It is interesting to note that the size of the '*THI* gene' observed is almost exactly those estimated for the 'average' gene within the yeast genome (Dujon, 1996), where the typical yeast gene is a 1450bp ORF, preceded by an upstream region of 309bp, and followed by a down-stream region of 163bp. In association with all four *THI* loci, is a region homologous to the 'YCR107w' gene of chromosome III. The homologous open reading frames on chromosome X and XIV appear to be capable of encoding a complete protein, whereas the chromosome VI ORF has diverged and mutated.

Complete sequencing of the yeast genome is indicating that multiple copies of many genes are present within the sixteen yeast chromosomes; the retention of complete functional genes may result from the continued 'selection' in *S. cerevisiae* for important phenotypes (Bork *et al.*, 1992; Koonin *et al.*, 1994; Melnick and Sherman, 1993). The question then arises as to why the yeast cell has evolved four copies of essentially the same gene, whereas the surrounding sequence has diverged between all four loci. The events and mechanisms by which '*THI5*' has been multiplied are discussed below. It is, however, apparent from the level of homology, that they either arose by a recent mutational event and there has not been enough time for deleterious mutations to accumulate or that a selection pressure is acting upon them, which maintains them as functional units. It should be noted that the *THI4* in

```
          ....................................|.............................
ScTHI5    MSTDKITFLLNWQPTPYHIPIFLAQTKGYFKEQGLDMAILEPTNPSDVTELIGSGKVDMGLKAM
ScTHI11   MSTDKITFLLNWQPTPYHIPIFLAQTKGYFKEQGLDIAILEPTNPSDVTELIGSGKVDMGLKAM
ScTHI12   MSTDKITFLLNWQPTPYHIPIFLAQTKGYFKEQGLDMAILEPTNPSDVTELIGSGKVDMGLKAM
ScTHI13   MSTDKITFLLNWQPTPYHIPIFLAQTKGYFKEQGLDIAILEPTNPSDVTELIGSGKVDMGLKAM

          ............................................................
ScTHI5    IHTLAAKARGFPVTSVASLLDEPFTGVLYLKGSGITEDFQSLKGKKIGYVGEFGKIQIDELTKH
ScTHI11   IHTLAAKARGFPVTSVASLLDEPFTGVLYLKGSGITEDFQSLKGKKIGYVGEFGKIQIDELTKH
ScTHI12   IHTLAAKARGFPVTSVASLLDEPFTGVLYLKGSGITEDFQSLKGKKIGYVGEFGKIQIDELTKH
ScTHI13   IHTLAAKARGFPVTSVASLLDEPFTGVLYLKGSGITEDFQSLKGKKIGYVGEFGKIQIDELTKH

          ........................|...................................
ScTHI5    YGMKPEDYTAVRCGMNVAKYIIEGKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLAC
ScTHI11   YGMKPEDYTAVRCGMNVAKYIIEDKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLAC
ScTHI12   YGMKPEDYTAVRCGMNVAKYIIEGKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLAC
ScTHI13   YGMKPEDYTAVRCGMNVAKYIIEGKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLAC

          ..........................................................|.........
ScTHI5    LGCCCFCTVLYICNDEFLKKNPEKVRKFLKAIKKATDYVLADPVKAWKEYIDFKPQLNNDLSYK
ScTHI11   LGCCCFCTVLYICNDEFLKKNPEKVRKFLKAIKKATDYVLADPVKAWKEYIDFKPQLNNDLSYK
ScTHI12   LGCCCFCTVLYICNDEFLKKNPEKVRKFLKAIKKATDYVLADPVKAWKEYIDFKPRLNNDLSYK
ScTHI13   LGCCCFCTVLYICNDEFLKKNPEKVRKFLKAIKKATDYVLADPVKAWKEYIDFKPQLNNDLSYK

          ............................................................
ScTHI5    QYQRCYAYFSSSLYNVHRDWKKVTGYGKRLAILPPDYVSNYTNEYLSWPEPEEVSDPLEAQRLM
ScTHI11   QYQRCYAYFSSSLYNVHRDWKKVTGYGKRLAILPPDYVSNYTNEYLSWPEPEEVSDPLEAQRLM
ScTHI12   QYQRCYAYFSSSLYNVHRDWKKVTGYGKRLAILPPDYVSNYTNEYLSWPEPEEVSDPLEAQRLM
ScTHI13   QYQRCYAYFSSSLYNVHRDWKKVTGYGKRLAILPPDYVSNYTNEYLSWPEPEEVSDPLEAQRLM

          ....................
ScTHI5    AIHQEKCRQEGTFKRLALPA
ScTHI11   AIHQEKCRQEGTFKRLALPA
ScTHI12   AIHQEKCRQEGTFKRLALPA
ScTHI13   AIHQEKCRQEGTFKRLALPA
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Figure 8.1: ClustalV alignment of primary peptide sequences from *THI* family members. All four genes encode for a protein 340 amino acid residues in length. Alignment of the three loci was by Clustal V (Higgins *et al.*, 1992), with a mismatch between the residues indicated as a ' | ' and identical resides as ' ∘ '. Each locus showed an ≈99% identity to the others, with a very highly conserved nature, all mutations result in a conserved or neutral substitution i.e. conserved substitutions; M:I, G:D, and neutral substitution, Q→R.

the associated thiazole precursor pathway, present as a single copy has an expression level 4-fold higher (as assessed by *lac*Z reporter gene fusion) to that of either *THI5* or *THI12* in the opposing pyrimidine precursor pathway (R. Burrows, personal communication). From these data, it is possible that multiple copies of the '*THI*-gene' are required to maintain a balanced flux within the thiamine biosynthetic pathway. However, the question of why four copies are required remains unanswered and requires further investigation.

So to the question, by what mechanism(s) have multiple copies of the '*THI*' gene been generated. All four genes, *THI5*, *THI11*, *THI12* and *THI13* are located in the sub-telomeric region of their respective chromosomes and are oriented with the same polarity with respect to the telomere. Telomeres have a conserved structure in most eukaryotic organisms, with a guanine (G)-rich terminal repeat, $TG_{1\text{-}3}$. The telomeres are thought to be involved in end to end fusion, and protecting against degradation in aiding complete replication of chromosomes. In addition to these functions, yeast telomeres have several interesting properties. They exhibit a non-nucleosomal chromatin structure, and show a transcriptional 'silencing' effect on adjacent genes. Also they show an apparent physical clustering and undergo non-random recombinational interactions (Klein *et al.*, 1992; Konkel, *et al.*, 1995). The majority of sequence redundancy in the yeast genome is also found within these sub-telomeric regions (within the last 25kb of each end), which are characterized by a low gene density, low transcription rates, low recombination and late replication. Within the sub-telomeric region are a variety of elements, which are thought to act as fillers for increasing chromosome size to some minimum threshold level necessary for chromosome stability. They also appear to serve as barriers against telomere induced transcriptional silencing, and are regions for adaptive amplification of genes. In addition, it has been suggested that they provide a secondary mechanism of telomere maintenance via recombination when telomerase activity is absent (Louis, 1995). The level of redundancy in the sub-telomeric regions, together with the variation in copy number and location of repeated sequence, indicates a degree of recombinational rearrangement. Thus, the sub-telomeric regions may be site(s) of gene amplification. Since any one telomere can replace any other functionally, translocations from duplicated regions within a telomeric region have no phenotypic consequence. Additionally, there are few genes located within the sub-telomeric regions and those present are usually multicopy. Hence, insertions which disrupt genes are unlikely to cause problems. Also as this region appears to be rapidly evolving, adaptation via gene amplification could be faster than in other locations within the yeast genome. Recombination events between telomeres provide a mechanism for amplification of genes in this region, where such amplification may offer the opportunity for a faster

rate of adaptation. This adaptation via gene amplification may explain the presence of multigene families which are under selective pressure i.e. *SUC*, *MAL*, *MEL* and *RTM* families (Louis, 1995).

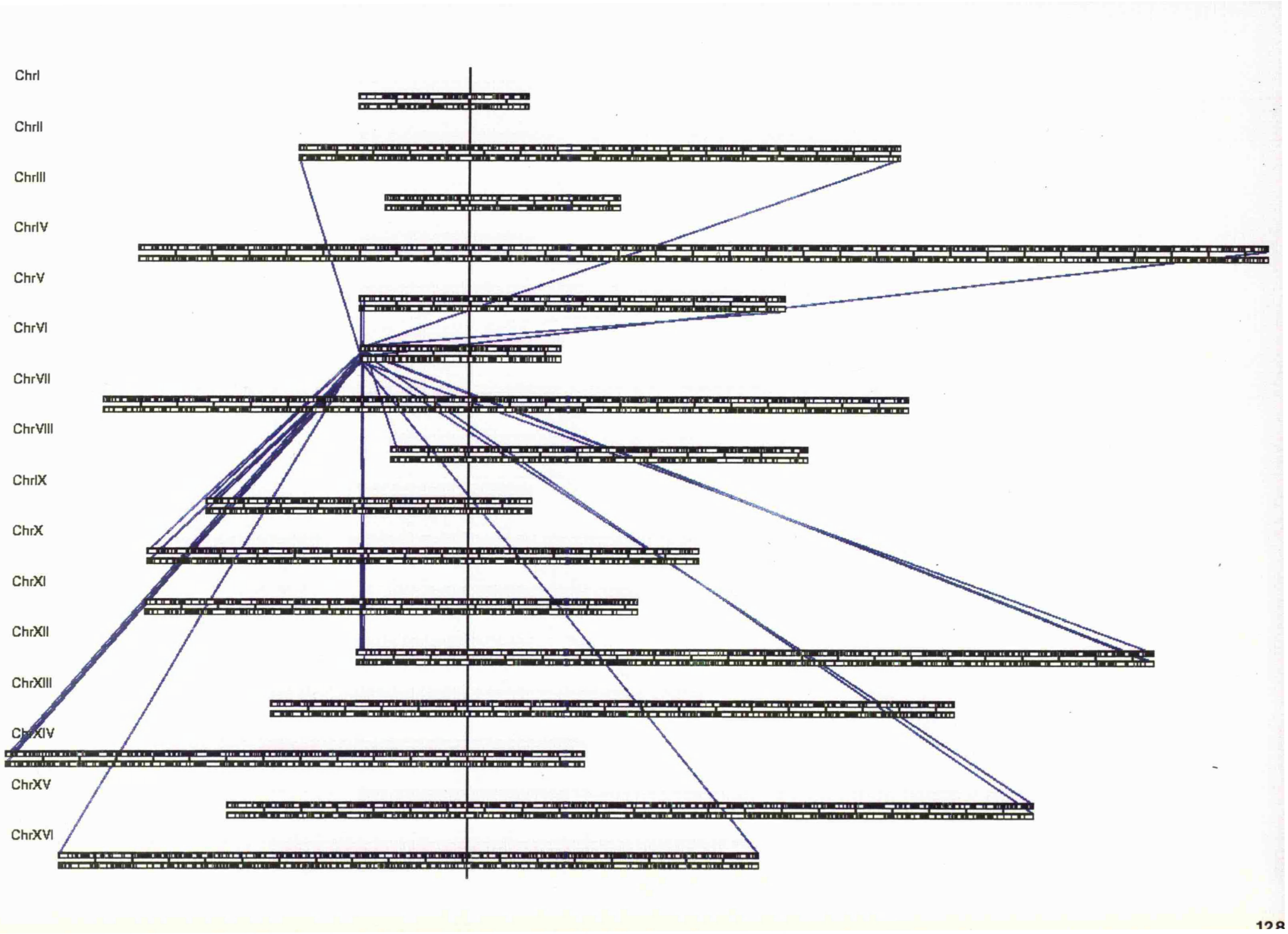
Another interesting feature to come from the completed yeast genome project, is that gene density is not uniform along the chromosomes. In most chromosomes, there are segments (several dozens of kilobases in length), in which gene density is higher than average. These segments of high gene density are separated by other regions (usually shorter) of a much lower gene density. In some chromosomes, the spacing between such regions is regular. The pericentric and sub-telomeric regions always show a low gene density. In the sub-telomeric regions, large segments (upto a few dozens of kilobases in length) containing several ORF's, are often identical between several chromosomes, suggesting recent or even continuous exchange of genetic information (Louis, 1995).

Comparison of DNA sequence for the yeast chromosomes, shows that there has been extensive duplication. From a comparison of the four telomeric regions containing the '*THI*' gene(s) (i.e. chrVI-L, chrXIV-L, chrX-R and chrIV-L), it is evident that a degree of mutation has occurred, where point mutations have resulted in the shifting in alignment. However, the DNA sequence retains its fidelity between these regions. Also there is a distinct region unique to both chromosome VI and XIV, which is not found within the chromosome X telomere, and appears to be an extension onto the chromosome VI and XIV sequence. The chromosome VI and XIV telomeres appear to be identical (if single point mutations are taken into account, which affect sequence alignment) over 20Kb, with the chromosome X telomeric region showing a similar level of identity to the chromosome VI telomere, however, the level of mutation would indicate that the recombinational event is not recent and has occurred within the yeast's evolutionary past. This can be illustrated by the analysis of homologous sequence using the Martinsried Institute for Protein Sequences (MIPS) 'Gene Browser' [1].

Chromosome VI when 'probed' against the other yeast chromosome DNA sequences (excluding telomeres, transposon elements, rDNA sequence and selection for ORF's), clearly shows a sequence homology from the left end of chromosome VI to chrIII-R, chrIV-L, chrX-R and chrXIV-L (see figure 8.2). Detailed examination of these chromosome regions and ORF homologies shows that the '*THI*-gene' is immediately adjacent (proximal to the telomere) to a gene encoding a putative aryl-alcohol dehydrogenase, YDL243c, YFL057/056c, YJR155w, YNL331c, which is

1. Copyright MIPS. Located at Universal Resource Locator, HTTP://speedy.mips.biochem.mpg.de/

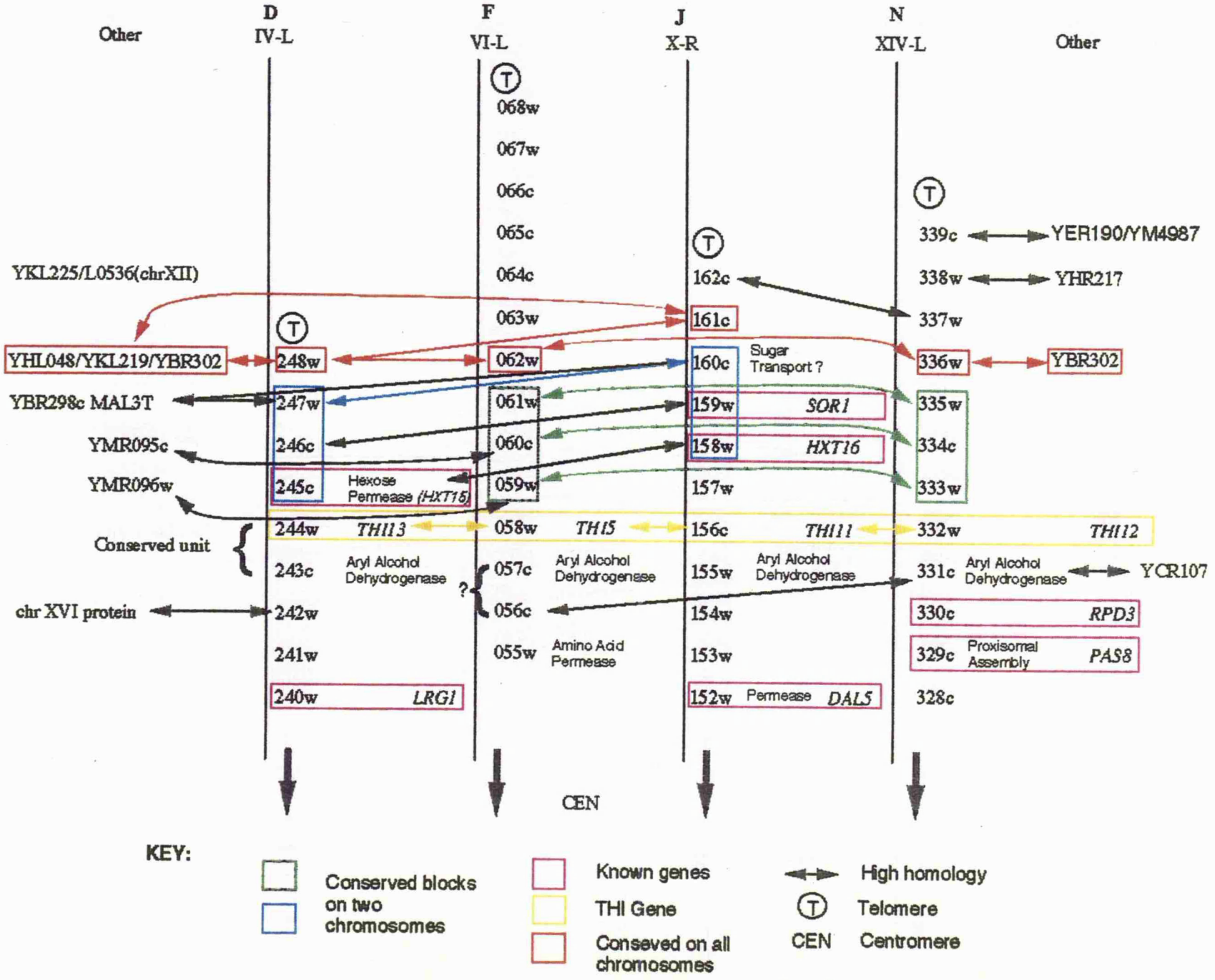
Figure 8.2: MIPS genome sequence similarity representation of chromosome homology. Sequence similarity relations identified within or across genomes are represented and visualised as a genomic sequence similarity graph (GSG). This allows for the easy identification of redundant genomic elements (e.g. telomeres, Ty's, deltas, t-RNAs, ORFs) by graph properties without knowledge of their individual structure. Global properties based on sequence similarities can be easily investigated, allowing the visualisation and evaluation of inter-chromosomal relationships. The GSG resolution and sensitivity were set as follows: Selection for ORF's only, with a protein homology index 500-600, and a DNA sequence homology 600-600. Protein homology from the left telomeric region of chromosome VI is indicated by solid blank lines. Open reading frames (+100 amino acid residues in length) are indicated on each chromosome.


ChrI
ChrII
ChrIII
ChrIV
ChrV
ChrVI
ChrVII
ChrVIII
ChrIX
ChrX
ChrXI
ChrXII
ChrXIII
ChrXIV
ChrXV
ChrXVI

also present on chromosome III (YCR107w). In relationship to the '*THI*-gene', it is apparent from this analysis, that there is a distinct grouping of genes associated on each of the different chromosomes. This grouping has been unilaterally replicated to one other of the four replicated sites. The chromosome VI site shows the same grouping of three ORF's as that from chromosome XIV, whereas chromosome IV has the same grouping as that of chromosome X (figure 8.3). It therefore appears, that chrIV and chrX co-evolved, as did chrVI and chrXIV, however, there must have been a common ancestor, from which the other replicated regions arose. Within the yeast genome, replication of gene clusters has been reported e.g. *ARC* and *COR* (Melnick and Sherman, 1993). Analysis of the right arm of chromosome XV, contains previously identified sub-telomeric repeat elements, with in addition two other members of repeated gene families (Pryde, Huckle and Louis, 1995). From the yeast genome project, the sequencing of the telomeric regions has revealed a great deal of shared homology between ends i.e. I right and VII right (de Steensma *et al.,* 1989; Johnston *et al.*, 1994); IX left and X left (Riles *et al.*, 1993; Louis *et al.*, 1994); III left and XI right (Dujon *et al.*, 1994); III right, XI left and XV right (Alexandraki and Tzermia 1994; Pyrde *et al.*, 1995). These mosaic regions are where the multicopy *SUC* (Carlson *et al.*, 1985), *MEL* (Naumov *et al.*, 1990, 1991) and *PHO* (de Steensma *et al.*, 1989; Johnston *et al.*, 1994) genes are found. These shared homologies are indicative of recombinational interactions among the different ends. However, in many cases no flanking homologies are seen adjacent to the repeats, making homologous recombination an unlikely process of the spread of blocks of homology (Pryde *et al.*, 1995). It has been recently reported that within the replicating yeast cell nucleus, telomeric clustering occurs (M. Gotta *et al.*, cited in Louis, 1995; Klein *et al.*, 1992; Konkel, *et al.*, 1995), where during mitotic division, the yeast chromosomes segregate and cluster to form foci within the cell nuclear membrane periphery, thus providing a close proximity for chromosome rearrangement. Such an event would allow the recombinational events that have resulted in the replication of the '*THI*-gene' and the associated gene cluster.

Another factor postulated to play a role in genome evolution and telomere elongation is retrotransposition, with the suggestion that chromosomes are continually extended over evolutionary time. Examination of chromosomes in transposition-induced cells, shows that multiple transposition events occur in the chromosomal DNA of virtually every cell that survives transposition induction (Boeke, Garfinkel and Styles, 1985). This increase in the number of transposons provides many more opportunities for recombination between the homologous elements, which can lead to gross genome rearrangement (Fink, Boeke and Garfinkel, 1986). Transposon (Ty)

Figure 8.3: Schematic representation of gene clusters. The relatedness between the different '*THI5*' family gene clusters on chromosomes IV, VI, X and XIV as predicted by MIPS (see text for details) are indicated. Open reading frames identified on each chromosome are indicated as their chromosome designation. Telomeres are indicated by T, with centromere shown by large arrows (CEN). The relatedness shown between DNA sequences (open reading frames) is indicated by double headed arrows. Possible and known gene functions are as indicated adjacent to each ORF. Sections of homology are indicated as colour blocks, and arrows indicate the possible interchange between the different chromosomes.


Other
D
IV-L
F
VI-L
J
X-R
N
XIV-L
Other
YKL225/L0536(chrXII)
YHL048/YKL219/YBR302
YBR298c MAL3T
YMR095c
YMR096w
Conserved unit
chr XVI protein
248w
247w
246c
245c
Hexose Permease (HXT15)
244w THI13
243c Aryl Alcohol Dehydrogenase
242w
241w
240w LRG1
068w
067w
066c
065c
064c
063w
062w
061w
060c
059w
058w THI5
057c Aryl Alcohol Dehydrogenase
056c
055w Amino Acid Permease
?
CEN
162c
161c
160c Sugar Transport ?
159w SOR1
158w HXT16
157w
156c THI11
155w Aryl Alcohol Dehydrogenase
154w
153w
152w Permease DAL5
339c YER190/YM4987
338w YHR217
337w
336w YBR302
335w
334c
333w
332w THI12
331c Aryl Alcohol Dehydrogenase YCR107
330c RPD3
329c Proxisomal Assembly PAS8
328c
KEY:
Conserved blocks on two chromosomes
Known genes
THI Gene
Conseved on all chromosomes
High homology
Telomere
CEN Centromere

elements which encode and express an active reverse transcriptase pose another potential threat to the cell. From, frequent reverse transcription of cellular mRNA, the resulting cDNA's, could act as a mutagen by recombination with the genomic homologue. As linear DNA is known to be highly recombinogenic in yeast, uncontrolled reverse transcription of cellular mRNA could lead to the accumulation of many processed pseudogenes. However, it should be noted that no such genes have yet been described in yeast. In the case of the *'THI'* family, no apparent mutagenic effect of a retrotransposition event is detectable and in fact all four *'THI'* regions show an intact coding and promoter sequence. Therefore, a retrotransposition event did not result in the replication of this family.

Genetic exchange occurs between repeated sequences that are present at different locations within the eukaryotic genome. This type of recombinational event has been ascribed important evolutionary roles, including the maintenance of sequence homogeneity in multigene families and the formation of duplications, deletions and chromosome rearrangements. Recombination between dispersed homologous sequences (ectopic recombination) occurs frequently during meiosis in *S. cerevisiae*, at levels similar to that of recombination between sequences at the same location on parental homologues (Klein and Petes, 1981; Lichten, Borts and Haber, 1987). The rate at which a sequence recombines is affected by its genomic location, and is reflected in the frequency of ectopic meiotic recombination. Recombinational events within the yeast sub-telomeric regions appear to be at a reduced level when compared to human and other organisms, which exhibit higher genome average levels of homologous recombination (de Steensma *et al.*, 1989). Indeed, meiotic double strand breaks are absent from the sub-telomeric regions, however, sequence comparisons have indicated that a great deal of ectopic exchange occurs over evolutionary time.

Both *THI5* and *THI12* are located within the left sub-telomeric region of their respective chromosomes, whereas both *THI11* and *THI13* are located within the right sub-telomeric region (see figure 8.3) on their respective chromosomes. What recombinogenic events have taken place that have resulted in the replication of the *THI5* gene between the four different chromosomes?. The level of identity shown between these four genes indicates that a selection pressure must be acting upon them or that they have only recently undergone the recombinogenic events resulting in their distribution. However, this latter option appears not to be the case as each gene is highly conserved and is flanked by regions which have undergone mutation. Of particular interest, is the idea of the telomere as a region for adaptive gene amplification, could this have resulted in the *THI5*, *THI11*, *THI12* and *THI13* genes?.

It has been reported that the sub-telomeric region does not undergo the same frequency of recombination as observed in other regions of the yeast chromosomes. Therefore, it is not possible to predict at what point within the evolutionary past that these sequences were formed and when they began to diverge. However, the mechanism by which these sequences replicated can be surmised. It would appear that the chromosome VI and XIV telomeric regions are most related and that these regions ectopically recombined from the chromosome X telomeric sequence, prior to the extension of the chromosome VI or XIV telomere. However, another possibility is that the chromosome X telomere is derived from either the chromosome VI or XIV telomere, which fragmented prior to recombination. The observed degree of divergence within the *THI5*, *THI11*, *THI12* and *THI13* primary amino acid sequences would indicate that there is a selective pressure acting upon them, and presumably that all four are active. Based upon the fact that efficiently expressed yeast genes show a marked bias in the use of particular codons (termed the codon bias index, which reflects the percentage of amino acid residues which show this bias e.g. the codon usage bias of *PGK* and *TRP1* are 0.92 and 0.31 i.e. highly biased and unbiased respectively), a calculated codon bias (CAI) for each of the *THI* genes of 0.547 (*THI5*), 0.532 (*THI11*), 0.538 (*THI12*) and 0.529 (*THI13*) would suggest that each is moderately expressed. The equivalent codon bias shown for all four '*THI*-genes' would seem to indicate that their evolutionary rate is equal. So does the yeast cell derive any advantage from having multiple copies of the '*THI*-genes'?, as it appears that all four are functional, this can only be answered by the experimental determination of the level of expression for each gene, which in turn would ascertain the role each plays within the yeast cell. The availability of multiple copies of one gene is advantageous, where a large amount of the gene product must be synthesized at a high rate in a relatively short period of time. This effect maybe observed within the '*THI*-family', where disruption of one locus is complemented by another. In conclusion, the '*THI*-gene' family of *THI5*, *THI11*, *THI12* and *THI13* appears to have been replicated by ectopic recombination of the telomeric region and not via retrotransposition, where the effect of the replication of this gene has yet to be determined. However, each gene appears to be maintained by a selective pressure, and must be advantageous to the yeast cell, as all four genes are highly conserved.

Addendum:

From recent sequence data (July 1996), it is now know that four copies of the *THI5* gene are present within the yeast genome. The fourth *THI5* homologue (termed *THI13*), 'YDL244w' is reported on the left telomere, chromosome IV (16,204 to 17,223 nt) (EMBL Accession No. Z74292). This homologue also shows a 99.5%

identity at the peptide level, to the other homologues (figure 8.1). Similar to *THI5*, 'YDL244w' is associated with 'YCR107w'. From this data, the observed 'Southern' hybridization patterns (chapter five), are consistent with four hybridization regions within the yeast genome, however, CHEF analysis indicated only three hybridization regions. This discrepancy is probably a result of a weak hybridization signal for the chromosome IV homologue. The evolution of this region of the chromosome IV, appears to be similar to the others, and has resulted in four dispersed regions within the yeast genome. In conclusion, there are four copies of the *THI5* gene, all of which appear to be functional, however, this has yet to be confirmed experimentally.

Chapter 9:
Materials and methods

9.1 Bacterial and Yeast strains

The genotype of the bacterial and yeast strains used in this study are shown in Table 9.1. The yeast strains S288Ca and PMY3 were obtained from Dr. P.A Meacock, at the Leicester Biocentre. Strain PMY3::*thi4* was obtained from Dr U. Praekelt, Leicester University. An isogenic series of yeast glucose repression mutants (RTY series) was obtained from Dr R. Trumbley, Medical college of Ohio, Ohio. The AM series of mutants deficient in cyclic AMP was obtained from Dr. A. Wheals, University of Bath. The yeast strain YPH149 (used in CHEF analysis) was obtained from Dr R. Mount, Biorad Ltd. Strains YPH499 and YPH500 were obtained from Dr P. Hieter, John Hopkins University, Maryland. All bacterial strains were obtained from Dr P.A Meacock at the Leicester Biocentre.

9.2 Vectors

The plasmid vectors used throughout this study are those from the pUC series (Vieira and Messing, 1982; Yanisch-Perron, Vieira and Messing, 1985) and pIC series (Marsh, Erfle and Wykes, 1984) of vectors. Yeast biosynthetic genes contained within the YDp plasmid series (Berben *et al.*, 1991) were used in the production of the gene disruption cartridge for 'one-step' gene disruption.

9.3 Culture media and supplements

All liquid media was sterilized by autoclaving at 15 p.s.i (121°C) for twenty minutes. All carbon sources were autoclaved separately at 10 p.s.i (115°C) for twenty minutes. Carbon sources were then added to a final concentration of 2%(w/v) to sterile liquid media after autoclaving. Bacterial strains were incubated at 37°C and yeast strains at 28°C.

Table 9.1: Bacterial and yeast strain genotypes

Bacterial strains

	Genotype	Reference
DH5αF'	*F'/ endA1*, *hsdR17*, (r_K^-, m_K^+), *supE44*, *thi-1*, *recA1*, *gyrA* (Nal^r), *relA1*, Δ(lacZYA-argF)U169, *deoR* (ϕ80*dlac*Δ(*lacZ*)*M15*)	(Woodcock *et al.*, 1989)
LE392	F^-, e14$^-$ (mcr^-), *hsdR514*, (r_K^-,m_K^+), *supE44*, *supF58*, *lacY1 or* Δ(*lac*IZY)6, *galK2*, *galT22*, *metB1*, *trpR55*	(Murray, Brammer and Murray, 1977)
NM522	*F'*, *lacI*^{q} , Δ(*lacZ*)*M15*, *proA*$^+$*B*$^+$/*supE*, *thi*, Δ(*lac-proAB*), Δ(*hsdMS-mcrB*)5(r_K^-,m_K^- McrBC$^-$)	(Gough and Murray, 1983)
JM83	F^-, *ara*, Δ(*lac-proAB*), *rpsL* (str^r), [ϕ80*dlac*Δ(*lacZ*)*M15*]	(Yanisch-Perron, Vieira and Messing, 1985)
XL1-Blue	*F'::Tn10 proA*$^+$*B*$^+$, *lacI*^{q}Δ(*lacZ*)*M15*/*recA1*, *endA1*, *gyrA96*(Nal^r), *thi*, *hsdR17* (r_K^-, m_K^+), *supE44*, *relA1*, *lac*	(Bullock, Fernandez and Short, 1987)

Yeast strains

S288Ca	*Mata*, *mal*, *gal2*, [cir+]	(Johnston and Mortimer, 1984) (Mortimer *et al.*, 1989)
PMY3a	*Mata*, *ura3-52*, *gal*+	(P.A Meacock, Personal communication.)
PMY3::*thi4*	*Mata*, *ura3-52*, *gal*+, *thi4*::URA3+	(Praekelt and Meacock, 1992)
RTY235	*Matα*, *his4-519*, *leu2,3-112*, *trp1-289*, *ura3-52*	(Williams and Trumbly, 1990)
RTY333	*Mata*, *leu2*, *trp1*, *ura3*, *cat1-42*(*snf1-42*)	(Williams and Trumbly, 1990)
RTY363	*Mata*, *his4-519*, *leu2,3,-112*, *ura3-52*, *cyc8*-Δ1::LEU2+	(Williams and Trumbly, 1990)
RTY418	*Matα*, *his4-519*, *leu2,3,-112*, *trp1-289*, *ura3-52*, *tup1*-Δ1::TRP1+	(Williams and Trumbly, 1990)
RTY438	*Matα*, *his4-519*, *leu2,3,-112*, *trp1-289*, *ura3-52*, *tup1*-Δ1::TRP1+, *cyc8*-Δ1::LEU2+	(Williams and Trumbly, 1990)
RTY474	*Mata*, *ade2-101*, *his3-200*, *leu2*-Δ1, *lys2-801*, *trp1*-Δ63, *snf1*-Δ100::URA3+	(Williams and Trumbly, 1990)
RTY508	*Mata*, *his4-519*, *leu2,3,-112*, *trp1-289*, *ura3-52*, *tup1*-Δ1::TRP1+, *snf1*-Δ100::URA3+	(Williams and Trumbly, 1990)
AM110-4C	*Matα*, *cyr1-2* (ts), *leu1*, *ade6*, *ade10*, *cam1*, *cam2*, *cam3*	(Matsumoto, Uno and Ishikawa, 1985)
AM180-2B	*Mata*, *bcy1*, *his7*, *ade6*, *ade10*, *cam1*, *cam2*, *cam3*	(Matsumoto, Uno and Ishikawa, 1985)
AM9-10A	*Matα*, *cyr1-1*, *bcy1*, *ade6*, *ade10*, *cam1*, *cam2*, *cam3*	(Matsumoto *et al.*, 1982)
YPH149	*Mata*, *ura3-52*, *lys2-801*, *ade2-101*, *his7*, *trp1Δ1* *CFVII*(*RAD2.p.YPH149*), [(*CFVII*(*RAD2.d.YPH146.TRP1*)]*r*-	(Gerring, Connelly and Hieter, 1991)
YPH499	*Mata*, *ura3-52*, *lys2-801*, *ade2-101*, *trp1*-Δ63, *his3*-Δ200, *leu2*-Δ1	(Sikorski and Hieter, 1989)
YPH500	*Matα*, *ura3-52*, *lys2-801*, *ade2-101*, *trp1*-Δ63, *his3*-Δ200, *leu2*-Δ1	(Sikorski and Hieter, 1989)

9.3.1 Bacterial Media

9.3.1.1 Luria-Broth (LB media) and Luria-Agar (L-Agar)

10.0g L^{-1} Tryptone (Difco), 5.0g L^{-1} Yeast extract (Difco), 5.0g L^{-1} NaCl
pH was adjusted to 7.2 with 10M NaOH and the medium sterilized by autoclaving. To form Luria agar medium, Difco agar was added to a final concentration of 2%(w/v), prior to sterilization.

9.3.1.2 TB top agar

10.0g L^{-1} Bacto-tryptone (Difco), 5.0g L^{-1} NaCl, 8.0g L^{-1} Bacto-agar (Difco).
The medium was then sterilized by autoclaving, the sterile molten agar was allowed to cool to 60°C, prior to the addition of sterile 1M $MgSO_4$ to a final concentration of 0.1M.

9.3.2 Yeast Media

9.3.2.1 Whickerham's chemical defined media (Wickerham, 1951)

1x Salts / Nitrogen base:
1g L^{-1} KH_2PO_4, 0.5g L^{-1} $MgSO_4.7H_2O$, 0.5g L^{-1} NaCl, 0.5g L^{-1} $CaCl_2.6H_2O$, 2.5g L^{-1} $(NH_2)_2SO_4$

Trace elements:
5mg ml^{-1} H_3BO_3, 4mg ml^{-1} $MnSO_4.4H_2O$, 4mg ml^{-1} $ZnSO_4.7H_2O$, 2mg ml^{-1} $FeCl_3.6H_2O$, 2mg ml^{-1} $Na_2MoO_4.2H_2O$, 1mg ml^{-1} KI, 0.4 mg ml^{-1} $CuSO_4.5H_2O$, were filter sterilized (using an Acrodisc® 0.2µM filter) and stored separately at 4°C.

Vitamins:
The following vitamins were filter sterilized separately (using an Acrodisc® 0.2µM filter) and then stored at 4°C.

Nicotinic acid	8.00mg ml^{-1}
Pantothenic acid	1.80mg ml^{-1}
Pyridoxine HCl	0.50mg ml^{-1}
Thiamine HCl	0.50mg ml^{-1}
Inositol	20.00mg ml^{-1}
Biotin	0.20mg ml^{-1}
p-aminobenzoic acid	0.50mg ml^{-1}
Riboflavin	0.09mg ml^{-1}

To 1L sterile 1x Salts/Nitrogen base the following were added: 100µl of each trace element, 1ml of each vitamin and glucose to a final concentration of 2%(w/v).

9.3.2.2 Yeast peptone (YEP) media

10g L^{-1} Yeast extract (Difco), 20g L^{-1} Bacto-peptone (Difco)

Sterile YEP media was supplemented with appropriate sterile carbon sources (stock concentration 40%(w/v)) to a final concentration of 2%(w/v). Agar plates were formed by the addition of agar (Difco) to a final concentration of 2%(w/v), prior to sterilization by autoclaving.

9.3.2.3 Pre-sporulation medium

8g L^{-1} Yeast extract (Difco), 3g L^{-1} Bacto-peptone (Difco), 20g L^{-1} Agar (Difco)

YEP media was supplemented with dextrose (stock concentration 40%(w/v)) to a final concentration of 10%(w/v).

9.3.2.4 Sporulation medium (Sherman, Fink and Lawrence, 1979)

10g L^{-1} Potassium acetate, 1g L^{-1} Yeast extract (Difco), 20g L^{-1} Agar, 0.5g L^{-1} Dextrose (Sigma).

A single pellet of NaOH, was added prior to autoclaving to prevent agar denaturation. After autoclaving, a $^1/_4$ amount of each amino acid required (see section 9.3.3.4) was added from sterile stock solutions.

9.3.2.5 Industrial molasses based medium

Blackstrap molasses is a by-product of the sugar extraction process, characterized by a polymorphic composition, varying in sugar composition/content and trace elements. Blackstrap molasses was supplied by Dr J. Hay, Distillers Company (Yeast) Ltd.

100g L^{-1} Black strap cane molasses,
2.0g L^{-1} $(NH_2)_2SO_4$, 2.0g L^{-1} KH_2PO_4, 0.6g L^{-1} $MgSO_4.7H_2O$

The medium was autoclaved at 10 p.s.i for twenty minutes and sediment was allowed to settle, before the clarified medium was decanted off under aseptic conditions. The clarified medium then was stored at room temperature, prior to use.

9.3.2.6 Synthetic-Dextrose (SD or YNB) medium

1.7g L^{-1} Yeast Nitrogen Base (YNB) -amino acids (Difco), 5g L^{-1} $(NH_4)_2SO_4$
SD medium was supplemented with the appropriate carbon sources (stock concentration 40%(w/v) to a final concentration of 2%(w/v). Amino acids were added to a final concentrations (see section 9.3.3.4) from sterile stock solutions as required. Prior to sterilization by autoclaving, agar (Difco) was added to a final concentration of 2%(w/v) to form solid media plates.

9.3.3 Medium supplements

9.3.3.1 Carbon source

D-glucose, D-fructose, D-raffinose, L-lactate, glycerol and sucrose were prepared as 40%(w/v) stock solutions in distilled water and sterilized by autoclaving at 10 p.s.i for ten minutes.

9.3.3.2 Antibiotics

Ampicillin:
Stock solutions of the sodium salt at 20 mg ml^{-1} were dissolved in sterile distilled water, filter sterilized using an Acrodisc® 0.2µM filter and stored at -20°C.

9.3.3.3 X-Gal, IPTG AMP media

L-Agar, 40µg ml^{-1} X-gal, 40µg ml^{-1} IPTG, 50µg ml^{-1} ampicillin
Media was prepared by mixing X-Gal, IPTG and ampicillin with molten L-agar at 55°C, pouring plates immediately. X-Gal was prepared as a 20mg ml^{-1} stock solution in N,N-dimethylformamide. IPTG and ampicillin were prepared as a 20mg ml^{-1} stock solutions in distilled water. Both solutions were sterilized by filter sterilization using a 0.2µM Acrodisc® filter and stored at -20°C.

9.3.3.4 Amino acids, bases and vitamins

	Stock	Storage
L-Arginine HCl	2mg ml^{-1}	4°C
L-Histidine HCl	2mg ml^{-1}	4°C
L-Leucine	3mg ml^{-1}	RT
L-Methionine	2mg ml^{-1}	4°C
L-Tryptophan	2mg ml^{-1}	4°C
Adenine sulphate	2mg ml^{-1}	RT
Uracil	2mg ml^{-1}	RT
Thiamine HCl	200µg ml^{-1}	4°C

Amino acids and vitamins were filter sterilized using an Acrodisc® 0.2μM filter and stored as indicated prior to use. Tryptophan was stored in a light-proof container at 4°C. To 100ml medium requiring supplementation, 1ml of the above solution(s) were added.

9.3.4 Yeast growth conditions

9.3.4.1 Shake flask cultures

Starter cultures of each yeast strain were inoculated in 5ml of the appropriate medium and incubated at 28°C, in an orbital shaker at 190 rpm for approximately eighteen hours. The density of the starter culture was determined using a haemocytometer, appropriate dilutions were made with the corresponding growth medium to a starting inoculum density of $1.0x10^6$ cells ml^{-1}. In shake-flask cultures, the initial medium:volume ratio was 1:5. Growth was allowed to continue at 28°C, 190 rpm in an orbital shaker. Within the series of growth phase experiments, cell growth was monitored hourly, after a cell density of $5.0x10^6$ cells ml^{-1} was reached. At this point 20ml yeast cell suspension samples were taken hourly and cells collected by centrifugation at 5,000 rpm for five minutes (4°C). Cell samples were stored at -20°C for further analysis.

9.3.4.2 Fermenter cultures

Two fermenter systems were used within this study; a LH Series-500 1L fermenter and an LH Series-2000 18L fermenter. The set-up procedures were as outlined in the appropriate manual. Initial fermenter studies were carried out in the LH Series-500 system, with the culture conditions as follows:

Temperature	26°C
Agitation	500 rpm
pH	no control
Aeration	100% DO_2

Dissolved oxygen was monitored using an Ingold polarographic electrode and maintained at 100% by mixed gas flow (sterile air/nitrogen). The Series-500 fermenter was sterilized containing the appropriate media, minus carbon source, by autoclaving (15 p.s.i for twenty minutes). Carbohydrate was added to a final concentration of 2%(w/v) and 100μl sterile antifoam-289 (Sigma) was added (to prevent foaming). The fermenter was run for two hours prior to inoculation, to allow complete mixing of the carbohydrate, antifoam and saturation of the media with oxygen.

A starter culture and shake flask culture were set-up and used to inoculate the fermenter to a starting density of 1.0-5.0x10^6 cells ml^{-1}. Cell growth was monitored hourly by both optical density (OD_{650}) and number of cells per ml (counted using a haemocytometer). Every two hours, 20ml yeast cell suspension was taken and cells collected by centrifugation; 1ml of the supernatant was also collected for carbohydrate analysis. Both samples were stored at -20°C for analysis.

The set-up procedure for the LH Series-2000, 18L fermenter was as outlined in the Series-2000 manual. The growth conditions used were as follows:

Temperature	26°C
Agitation	500 rpm
pH	no control
Aeration	no control
Air flow	10L min^{-1}

The same procedures were employed as outlined for the LH Series-500 fermenter, except sterilization was carried out *in situ* at 15 p.s.i for twenty minutes. Appropriate carbon source addition was to a final concentration of 2%(w/v). Antifoam-289 (Sigma) was also added to reduce foaming. Inoculation and sampling were as outlined for the Series-500 fermenter.

9.3.5 Yeast Mating and tetrad dissection (Spencer and Spencer, 1990)

The protocol for constructing a new yeast strain involves four distinct stages. The first stage involves the formation of a diploid by mass mating using an 'drop overlay' technique, where two haploid strains of opposite mating type are mated, followed by a sporulation stage, where the diploid strain is induced to form spores. These spores are then isolated and the ascus wall removed from the tetrad. The final stage involves the removal of the four haploid spores from a single tetrad and their specific positioning onto a nutrient agar plate prior to subsequent studies.

Diploids were constructed by mating strains of opposite mating type onto the surface of selective agar plates (SD medium - appropriate amino acid i.e. one yeast strain is auxotrophic for amino acid X, whereas the other mating strain is auxotrophic for amino acid Y, so on SD -X,-Y the diploid strain is selected for). The different mating strains were grown overnight in liquid YEPD medium. Mass

mating of these two different haploid strains was by an 'drop overlay' technique, where 50µl of culture from one mating type was spotted onto the selective medium and allow to air dry, followed by a repeat spotting with the other mating type. Incubate at 28°C for three to five days. This 'spot mat' of yeast colonies was then streaked onto SD selective media for both auxotrophic markers, to isolate individual colonies and incubated at 28°C for five days. Isolated individual colonies were transferred onto pre-sporulation medium and grown at 28°C for three days. Single colonies were then streaked onto sporulation medium and incubated at 25°C for four days.

Tetrads were visualized by suspending a small number of cells in sterile distilled water and examination under a magnification of 250x to 400x. Spores were then transferred from the sporulation plate using a sterile toothpick into 100µl sterile distilled water and vortexed vigorously. The ascus wall was removed by enzymatic digestion with ß-glucuronidase (500u) (Sigma) at room temperature for ten minutes, incubation was continued at room temperature until digestion was complete. The reaction was then stored on ice prior to streaking the treated spore suspension in a parallel line onto a YEPD dissection plate. Tetrads were dissected with a Singer Instruments MSM™ system dissection microscope as outlined in the supplied manual (see also (Ausubel *et al.*, 1989)). Dissected spores were then grown on YEPD agar medium at 28°C for three to five days, prior to analysis.

9.4 Standard buffers, solutions and enzymes

1x TE buffer (pH8.0):

1mM EDTA (pH8.0), 10mM Tris-HCl (pH8.0)

10x TAE buffer (pH8.0):

48.4g L^{-1} Trizma base, 11.2ml L^{-1} glacial acetic acid, 4.7g L^{-1} EDTA

10x TBE buffer (pH8.0):

104.0g L^{-1} Trizma base, 55.0g L^{-1} Boric acid, 9.3g L^{-1} EDTA

10x MOPS:

41.8g L^{-1} MOPS, 6.8g L^{-1} sodium acetate.$3H_2O$, 1.9g L^{-1} Na_2EDTA

pH was adjusted to 7.0 with 10M NaOH, sterilized by autoclaving at 10 p.s.i for twenty minutes and stored at 4°C.

20x SSC:

175.3g L^{-1} NaCl, 88.2g L^{-1} sodium citrate

RNase-DNase free:

Ribonuclease A (Sigma) was prepared as a 10mg ml^{-1} stock solution in 10mM Tris-HCl (pH7.5), 15mM NaCl. DNase activity was destroyed by heating at 105°C for ten minutes and the solution was cooled on ice before individual aliquots of 100µl were dispensed and stored at -20°C. RNaseA was used at a concentration of 1mg ml^{-1}.

Gel loading buffers:

DNA gel loading buffer:
0.25%(w/v) bromophenol blue (Sigma), 0.25%(w/v) xylene cyanol (Sigma), 40%(w/v) sucrose in water. Sterilize by autoclaving at 15 p.s.i for twenty minutes.

RNA gel loading buffer:
50%(v/v) glycerol, 0.4%(w/v) bromophenol blue (Sigma), 0.4%(w/v) xylene cyanol (Sigma), 1mM EDTA (pH8.0). Sterilize by autoclaving at 15 p.s.i for twenty minutes.

A 10x stock solution of each loading buffer was used and $^{1}/_{10}{}^{th}$ volume stock solution was added to either DNA or RNA samples prior to analysis on an agarose gel (see section 9.8.6 or 9.8.9 for DNA or RNA gel analysis, respectively).

Phenol/CHCl3/IAA:

Phenol was supplied as a liquid equilibrated with 100mM Tris-HCl (pH7.6) from Fisons chemicals Ltd and was used in the deproteinization of DNA and RNA preparations. An equilibrated 24:1 ratio CHCl3/ IAA (v/v) was added to an equal volume phenol forming a 25:24:1 (v/v/v) ratio and was stored in a light proof container at 4°C.

9.5 Methods for DNA isolation

9.5.1 Plasmid DNA isolation from *Escherichia coli*

9.5.1.1 Preparation of plasmid DNA by CsCl density centrifugation (Maniatis *et al.*, 1982)

An *E. coli* transformant was grown under antibiotic selection, overnight in 400ml LB medium. Cells were harvested by centrifugation at 6,000 rpm for ten minutes in a Sorvall® GSA rotor.

The cell pellet was resuspended in 3.5ml sucrose solution (25%(w/v) sucrose, 0.05M Tris-HCl (pH8.0)) and chilled on ice. 0.6ml lysozyme solution (10mg ml^{-1} in 1x TE (pH8.0)) was added and incubated on ice for fifteen minutes. The enzyme was inactivated by the addition of 1ml 0.5M EDTA (pH8.0), followed by a further incubation on ice for fifteen minutes. To the lysate 4.5ml Triton x100 solution (2%(v/v) triton x100, 0.05M Tris-HCl (pH8.0), 0.0625M EDTA (pH8.0)) was added and mixed via gentle inversion. The resultant lysate was clarified by centrifugation at 19,000 rpm (4°C) in a Sorvall® SS-34 rotor for thirty minutes. To 8ml clarified supernatant, 7.9g CsCl (Analar™) and 0.5ml ethidium bromide (10mg ml^{-1}) was added. After all CsCl had dissolved, the solution was centrifuged at 15,000 rpm (20°C) in a Sorvall® SS-34 for thirty minutes. The resultant supernatant was transferred into TFT65.13 'ultracrimp' polyallomer tubes (DuPont), prior to centrifugation at 40,000 rpm (20°C) for forty hours, under vacuum in a Sorvall® TFT65.13 rotor.

The plasmid containing fraction was extracted with an equal volume of iso-propanol equilibrated with CsCl saturated 1x TE buffer (pH8.0). This extraction was repeated until no ethidium bromide was present in the upper aqueous phase. The plasmid containing aqueous phase was dialysed at room temperature against 1x TE (pH8.0), with fresh 1x TE buffer every hour, for five to six hours, to remove CsCl from the plasmid containing fraction. The resultant DNA suspension was stored at either 4°C or at -20°C. DNA concentration was determined by absorption measurement on a Shimadzu spectrophotometer at 260nm.

9.5.1.2 Preparation of plasmid DNA by alkaline lysis

Plasmids were prepared by the following method based upon that of (Birnboim and Doly, 1979).

An overnight culture of *E. coli* containing the plasmid of interest was grown under antibiotic selection in a 100ml of LB-media. Bacterial cells were harvested by centrifugation at 5,000 rpm for ten minutes in a Sorvall® GSA rotor. The cell pellet was resuspended in 1ml cold lysis buffer (20mM Tris-HCl (pH8.0), 50mM glucose, 10mM EDTA (pH8.0), 2mg ml^{-1} lysozyme) and incubated on ice for thirty minutes. 2ml freshly made 0.2M NaOH/1%(w/v) SDS was added, followed by a further incubation on ice for five minutes. 1.5ml 3M sodium acetate (pH5.5) was added and the suspension held on ice for one hour. The lysate was clarified by centrifugation at 10,000 rpm (4°C) for twenty minutes in a Sorvall® SS-34

rotor. Two volumes ice-cold 100% ethanol (v/v) were added to 4ml cell lysate and the nucleic acids precipitated at -20°C for thirty minutes, followed by centrifugation at 10,000 rpm (4°C) for ten minutes in a Sorvall® SS-34 rotor.

Nucleic acids were resuspended in 450µl sterile 1x TE buffer (pH8.0), extracted twice with an equal volume of phenol/$CHCl_3$/IAA and precipitated using sodium acetate/ethanol, prior to resuspension in 200µl 1x TE buffer (pH8.0). To the resuspended nucleic acids, RNase A (DNase free) was added to a final concentration of 20mg ml^{-1} and incubated at 37°C for one hour. Plasmid DNA was then precipitated by sodium acetate/ethanol, at room temperature for ten minutes, followed by centrifugation at 13,000 rpm for ten minutes in a bench-top microfuge. The DNA pellet obtained was resuspended in 200µl sterile distilled water and stored at -20°C.

9.5.1.3 Preparation of plasmid DNA by phenol extraction ('miniprep') (Serghini, Ritzenthaler and Pinck, 1989)

A modified protocol of Serghini, Ritzenhaler and Pinck (1989) was used to screen possible recombinant *E. coli* containing plasmid DNA.

2ml cultures of *E. coli* were grown overnight under appropriate antibiotic selection. 1.5ml cells were harvested by centrifugation at 13,000 rpm at room temperature for two minutes in a bench-top microfuge. The supernatant was discarded and the cell pellet resuspend in 50µl TNE buffer (10mM Tris-HCl (pH8.0), 100mM NaCl, 1mM EDTA (pH8.0)). An equal volume of phenol/$CHCl_3$/IAA was added and the cell suspension vortexed vigorously for thirty seconds, followed by incubation at room temperature for two minutes and repeated vortex. Cell debris was removed by centrifugation at 13,000 rpm for ten minutes in a bench top microfuge.

The upper aqueous phase was removed and the nucleic acids precipitated by sodium acetate/ethanol precipitation. The nucleic acid pellet obtained was washed with 70%(v/v) ethanol and resuspended in 48µl sterile distilled water, followed by storage at -20°C. Prior to analysis by restriction endonuclease digestion, RNase A (DNase free) was incorporated to a final concentration of 0.05µg μl^{-1} into the reaction mix containing the plasmid DNA.

9.5.2 Yeast genomic DNA isolation

9.5.2.1 Large scale preparation from yeast sphaeroplasts

An 800ml overnight yeast culture was harvested by centrifugation, using a Sorvall® GS3 rotor at 6,000 rpm for six minutes (20°C). The cell pellet was resuspended in 25ml 500mM EDTA (pH7.5) and the centrifugation step repeated. Cells were resuspended in 20ml distilled water, 2ml 0.5M EDTA (pH9.4) and 0.5ml 14.3M ß-mercaptoethanol (Sigma) and incubated at room temperature for twenty minutes. Cells were then harvested by centrifugation at 6,000 rpm for one minute (20°C) in Heraeus bench top centrifuge and resuspended in 20ml SE buffer (1.2M sorbitol, 0.1M EDTA (pH7.9)). 5mg yeast lytic enzyme (Novozym™) (5mg ml^{-1} in SE buffer) was then added, followed by incubation at 37°C for forty minutes with gentle agitation. Sphaeroplast formation was monitored by examination under a microscope.

Sphaeroplasts were harvested by centrifugation at 4,000 rpm for five minutes, washed in 20ml SE buffer and recovered by repeated centrifugation. The supernatant was discarded and the sphaeroplasts were resuspended in 10ml 0.15M NaCl, 0.1M EDTA (pH7.5) plus 2ml proteinase K (2mg ml^{-1}) and 0.5ml 20%(w/v) SDS, by gentle inversion. Genomic DNA was released from this sphaeroplast suspension by gentle inversion, at 37°C for two hours. Proteinase K was inactivated by incubation at 60°C for thirty minutes, cooled to room temperature and extracted with an equal volume $CHCl_3$/IAA (24:1 v/v). After centrifugation at 10,000 rpm for twenty minutes in corex™ tubes, the 'cloudy' aqueous phase containing the genomic DNA was precipitated with two volumes 100% ethanol (v/v). Genomic DNA was spooled at the interface with a sealed pasteur pipette and resuspended in 5ml 0.1x SSC, overnight at 4°C. 0.25ml 20xSSC was then added and contaminating RNA removed by incubation at 37°C for two hours with 25µl RNase A (DNase free) (10mg ml^{-1}). Genomic DNA was re-precipitated with ethanol, spooled and resuspended overnight in 0.5ml 0.1x SSC. After resuspension, 0.5ml 1x TE buffer (pH8.0) was added and the genomic DNA stored at 4°C. The concentration of genomic DNA was determined by absorption measurement at 260nm with a Shimadzu spectrophotometer.

9.5.2.2 Small scale preparation by glass bead homogenization (Hoffman and Winston, 1987)

A rapid small scale genomic DNA isolation method was used in the screening of disruption mutants, based upon the protocol described by (Hoffman and Winston, 1987).

An overnight 10ml culture of the chosen yeast strain was grown in YEPD medium. Yeast cells were collected by centrifugation at 5,000 rpm for five minutes and resuspended in 0.5ml sterile distilled water, followed by centrifugation at 13,000 rpm for ten seconds. Cells were then resuspended in 200µl Triton/SDS solution (2%(v/v) Triton x100, 1%(w/v) SDS, 100mM NaCl, 10mM Tris-HCl (pH8.0), 1mM EDTA) and an equal volume phenol/$CHCl_3$/IAA added. 0.3g sterile glass beads (2µM diameter) were added and cells were disrupted by vortexing for four minutes. 200µl sterile 1x TE (pH8.0) was then added and cell debris was removed by centrifugation at 13,000 rpm for five minutes (in a bench-top microfuge) at room temperature.

To the upper aqueous layer 1.0ml 100%(v/v) ethanol was added and nucleic acids were collected by centrifugation at 13,000 rpm for two minutes. The nucleic acids were then resuspended in 400µl 1x TE (pH8.0). Contaminating RNA was removed by incubation at 37°C for twenty minutes with RNaseA-DNase free (10mg ml^{-1}) at a final concentration of 0.025µg μl^{-1}. Genomic DNA was precipitated with 10µl 4M ammonium acetate/1.0ml 100%(v/v) ethanol and collected by centrifugation at 13,000 rpm for two minutes. Recovered genomic DNA was air dried for five minutes and resuspended in 50µl sterile 1x TE (pH8.0). 10µl of the above DNA suspension was then used in restriction endonuclease analysis.

9.5.3 Size fractionation of partial DNA digest on a sucrose density gradient

Partial digested genomic DNA was size fractionated on a continuous sucrose density gradient (Ausubel *et al.*, 1989). 200µg partial digested genomic DNA was loaded onto a 34ml volume, 10%-40% (v/v) linear sucrose gradient in 1M NaCl, 20mM Tris-HCl (pH8.0), 5mM EDTA (Hadfield, 1987). The partial digest was resolved by centrifugation at 25,000 rpm (15°C) for twenty four hours in a Sorvall® AH627 swing-out rotor. The gradient was fractionated into 1ml aliquots and fractions analysed for size of DNA fragments by agarose gel electrophoresis. Samples showing the correct sized fractions were then precipitated using sodium acetate/ethanol precipitation and resuspended in 100µl sterile distilled water.

9.6 Methods of RNA isolation from *Saccharomyces cerevisiae*

9.6.1 Phenol extraction (Praekelt and Meacock, 1990)

Yeast cell suspension (20ml) from growth analysis cultures was centrifuged at 5,000 rpm for five minutes and the cell pellet resuspended in 2ml 'Phenol' buffer (1%(w/v) tri-iso-propylnapthalene sulphonate (Kodak), 6%(w/v) 4-aminosalicylate, 50mM Tris-HCl (pH8.3), 6%(v/v) phenol equilibrated with 50mM Tris-HCl (pH8.3)). 300µl sterile glass beads were then added, the cells were vortexed until completely disrupted and then extracted three times with phenol:chloroform:iso-amyl alcohol (25:24:1 v/v/v). Nucleic acids were precipitated with $^{1}/_{10}^{th}$ volume 3M sodium acetate (pH5.3), two volumes 100% ethanol (v/v), at -70°C for one hour, collected by centrifugation and resuspended in one volume sterile distilled water (450µl).

Total cell RNA was precipitated by the addition of an equal volume of 6M lithium acetate (pH6.0), followed by incubation on ice for one hour. RNA was then collected by centrifugation, washed with 3M lithium acetate (pH6.0) and resuspended in an appropriate volume of sterile distilled water. Total cell RNA was then stored at -20°C prior to analysis.

9.6.2 Freeze-thaw method after (Schmitt, Brown and Trumpower, 1990)

From yeast cell suspensions (20ml), cells were collected by centrifugation at 5,000 rpm for five minutes at room temperature. The cell pellet was then resuspended in 1ml AE buffer (50mM sodium acetate (pH5.3), 10mM EDTA), 80µl 10% (w/v) sodium lauryl sulphate was added to 400µl cell suspension and vortexed for thirty seconds. An equal volume phenol equilibrated with AE buffer was then added and the vortex repeated. Cell disruption was by repeated incubation at 65°C for four minutes and freezing at -70°C for three minutes, for two freeze/thaw cycles. Cell debris was then removed by centrifugation at 13,000 rpm for two minutes and the upper aqueous layer was extracted, twice with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1 v/v/v). Total cell RNA was then precipitated with $^{1}/_{10}^{th}$ volume 3M sodium acetate (pH5.3) and two volumes 100%(v/v) ethanol at -70°C for one hour. The precipitated RNA pellet was washed once with 70%(v/v) ethanol and resuspend in 50µl sterile distilled water. Total cell RNA samples were then stored at -20°C prior to analysis.

9.7 Transformation procedures

9.7.1 *Escherichia coli* transformation

The modified protocol of (Lederberg and Cohen, 1974; Mandel and Higa, 1970) for competent *E. coli* cells after (Pritchard and Holland, 1985) was used.

LB media (10ml) was inoculated and incubated overnight at 37°C, with agitation (approximately 200 rpm). This culture was used to inoculate 100ml LB media in a 500ml conical flask and was grown to an optical density of 0.6 (OD_{650}). The culture was then chilled on ice and bacterial cells were harvested by centrifugation at 5,000 rpm for five minutes (4°C). Cells were resuspended in $^1/_2$ volume 10mM $MgCl_2$ (4°C) and the centrifugation step repeated. Collected cells were then resuspended in a $^1/_2$ volume 0.1M $CaCl_2$ (4°C) and held on ice for thirty minutes. Competent cells were then collected by centrifugation at 3,000 rpm for five minutes (4°C), resuspended in $^1/_{20}{}^{th}$ volume 0.1M $CaCl_2$ and stored on ice prior to use. Competent *E. coli* cells were stored upto three months at -70°C by addition of 210µl freezing mix (50%(v/v) glycerol, 0.1M $CaCl_2$) to 70µl competent cells.

To a DNA ligation reaction mix, 100µl 1x SSC, 200µl competent *E. coli* cells were mixed and incubated on ice for thirty minutes with occasional agitation. This cell/ligation mix was then heat shocked at 42°C for two minutes and incubated for twenty minutes on ice, without agitation. 2ml LB media was then added to each transformation mix and incubated at 37°C for sixty minutes with agitation. Transformed cells were then collected by centrifugation at 3,000 rpm for six minutes (4°C) and resuspended in 100µl LB media. 35µl transformed cells were plated onto selective media and incubated overnight at 37 °C.

9.7.2 Yeast transformation by alkali cation after (Ito *et al.*, 1983)

The yeast transformation protocol used was based upon that of (Ito *et al.*, 1983), as modified by (Mount, Jordan and Hadfield, 1996). All reagents were detergent free and sterile plastic-ware was used in all manipulations. Media was prepared and stored in detergent free bottles. All reagents used were sterilized by autoclaving prior to use.

An overnight yeast culture in 10ml YEPD was grown at 28°C, 190 rpm to a final density of 1.0x 10^7 cells ml^{-1}. Cells were harvested by centrifugation at 4,000 rpm for five minutes (20°C) and resuspend in 10ml transformation 1x TE (detergent free 10mM Tris-HCl (pH7.6), 0.1mM EDTA (pH7.6)). The centrifugation step was then repeated. The cell pellet was resuspended in 10ml LA buffer (0.1M lithium acetate in transformation 1x TE) and incubated at 28°C with gentle agitation for one hour. Competent cells were collected by centrifugation at 3,000 rpm for five minutes (10°C) and resuspended in 2ml LA buffer.

Transformation of competent yeast cells was either with plasmid DNA or linearised plasmid DNA fragments, for 'one-step' gene disruption (Rothstein, 1983). Plasmid DNA (0.1-10µg in a total volume ≤50µl) was transformed into competent yeast cells in the following reaction mix; 300µl competent yeast cells, 700µl PEG solution (50%(w/v) solution of PEG4000 in sterile distilled water, filter sterilize (0.2µM) made fresh as required) and mixed by gentle inversion. This transformation mix was then incubated at 30°C without agitation for one hour, followed by heat shocked at 42°C for fifteen minutes. YEPD (4ml) was added and the transformation mixture incubated for two hours at 28°C, 190 rpm in an orbital shaker. Transformed cells were collected by centrifugation at 3,000 rpm for five minutes (10°C) and the cell pellet resuspended in 300µl sterile distilled water. 100µl transformed cells were grown on appropriate selective agar medium and incubated at 28°C for three to five days.

9.8 DNA manipulations

9.8.1 Restriction endonuclease digestion

DNA (bacteriophage, genomic or plasmid) was digested in a total reaction volume of between 20µl to 100µl. A $^1/_{10}{}^{th}$ volume of appropriate restriction endonuclease buffer (10x), as supplied with the individual restriction endonuclease, was added to the reaction mix and the restriction endonuclease added at 10 units per µg DNA. This reaction mix was incubated at 37°C (unless otherwise stated) for at least one hour. Restriction endonucleases were obtained from either New England Biolabs (NEB) or BRL Ltd. Double digests were carried out either as separate digestion's followed by phenol/$CHCl_3$/IAA extraction and Na-acetate/ethanol precipitation or by combined digestion in an appropriate REact™ (BRL Ltd) or NEB™ (New England Biolabs) buffer which shows an optimum enzyme activity (see individual BRL or NEB catalogues for activity tables).

9.8.2 Removal of the 5' phosphate group from DNA restriction generated fragments by alkaline phosphatase treatment

Calf intestinal alkaline phosphatase (BCL molecular biology grade) was used to remove the 5' phosphate group from restriction endonuclease cut DNA, to prevent re-ligation. 1µg cut DNA in 1x CIAP buffer (total reaction volume of 30µl) was treated with 0.03u alkaline phosphatase and incubated at 37°C for twenty minutes. The reaction volume was increased upto 50µl with sterile distilled water and extracted once with an equal volume of phenol/$CHCl_3$/IAA. Phosphatase treated DNA was then precipitated using Na-acetate/ethanol at -70°C for one hour, washed with 70%(v/v) ethanol and resuspended in 10µl sterile distilled water.

9.8.3 Formation of blunt ends from sticky ends

9.8.3.1 Klenow fragment: 'Fill-in' of recessed 3' ends

To 1µg endonuclease cut DNA in a final volume of 10µl, a $^2/_{10}{}^{th}$ volume 10x nick translation buffer (0.5M Tris-HCl (pH7.2), 0.1M $MgSO_4$, 1mM DTT, 500µg ml^{-1} BSA), 1µl 2mM dNTP's (Pharmacia ultra-pure), 7µl sterile distilled water and 2u 'Klenow' (Large fragment DNA polymerase I) fragment (BRL Ltd) were added. The reaction mix was incubated at room temperature for thirty minutes and stopped by the addition of 1µl 0.5M EDTA (pH8.0). The reaction mix was extracted once with phenol/$CHCl_3$/IAA, followed by Na-acetate/ethanol precipitation. The recovered DNA was resuspended in an appropriate volume of sterile distilled water.

9.8.3.2 T4 DNA polymerase: Removal of 3' protruding termini

1µg restriction endonuclease cut plasmid DNA was treated in 30µl 1x T4 polymerase buffer (0.33M Tris-acetate (pH7.9), 0.66M K-acetate, 0.1M Mg-acetate, 5mM DTT, 1mg ml^{-1} BSA), 1.5µl dNTP mix (stock solution 2mM dATP, dTTP, dCTP and dGTP in sterile distilled water), 4u T4 DNA polymerase (BRL Ltd) and incubated at 37°C for five minutes. The reaction was stopped by the addition of 1.5µl 0.5M EDTA (pH8.0) and the reaction volume was increased to 50µl with sterile distilled water. An equal volume phenol/$CHCl_3$/IAA was added and extracted once. DNA was recovered by precipitation using Na-acetate/ethanol and resuspended in an appropriate volume of sterile distilled water.

9.8.4 Ligation of DNA fragments

Critical parameters within the ligation reaction are the concentration of DNA fragments and their length (King and Blakesley, 1986). DNA ligation and subsequent transformation was found to be increased by the addition of PEG8000 in the reaction mix (King and Blakesley, 1986). Another factor in the ligation reaction is the insert to vector ratio, where a range of 1:3 to 3:1 produces a 5 to 7 fold variation in transformants (Blakesley, 1986). The amount of DNA used in each ligation mix was determined by comparison with known amounts of restriction endonuclease cut plasmid DNA on a 0.8%(w/v) agarose minigel (see section 9.8.6). Using this information a series of ligation mixes, with 1:1, 1:3 and 3:1 insert:vector were set up in the following reaction mix:

Vector DNA	x µl
Insert DNA	y µl
T4 DNA ligase	1.0 µl
T4 buffer (x5)	2.0 µl
Distilled water	upto 10.0 µl

T4 DNA ligase (8u/µl) (Pharmacia) and T4 DNA ligase buffer (0.25M Tris-HCl (pH7.6), 50mM $MgCl_2$, 5mM ATP, 5mM DTT, 25%(w/v) PEG8000) (BRL Ltd) were used in all ligation reactions.

Ligation reactions were incubated at room temperature (≈26°C) for between four and six hours for 'sticky' end ligation, or overnight (approximately eighteen hours) at 15°C for both 'sticky' and 'blunt' end reactions. The above 10µl ligation mix was used in the transformation of competent *E. coli*. It is important to dilute the volume of ligation mix by at least a five fold dilution prior to the addition of competent cells, in order to prevent inhibition of transformation (King and Blakesley, 1986).

9.8.5 Ethanol precipitation of DNA

Nucleic acids were precipitated using sodium acetate and ethanol. To one volume DNA suspension, a 0.1 volume 3M sodium acetate (pH5.5 or pH7.0) was added, followed by two volumes ethanol (100%(v/v) at -20°C). The mixture was placed in a dry-ice/IMS (Industrial Methylated Spirit) bath (-70°C) for between thirty and sixty minutes and the precipitated nucleic acid was collected by centrifugation at 13,000 rpm for ten minutes in a bench-top microfuge. The nucleic acid pellet was then washed with ice-cold 70%(v/v) ethanol, the centrifugation step repeated and the nucleic acid resuspended in an appropriate volume of sterile distilled water or 1x TE (pH8.0).

9.8.6 Electrophoresis of DNA

Analysis of DNA was by electrophoresis on horizontal agarose gels. Varying the percentage of agarose within the gel allowed selective fractionation of DNA fragments (Maniatis *et al.*, 1982). Typical analysis was carried out on 0.8%(w/v) agarose (Seakem ME™) in 1x TAE, containing ethidium bromide (0.5µg ml^{-1}). Electrophoresis of samples was at a constant voltage between 50v and 100v (typically at 80v), until migration of the gel loading buffer (see section 9.4.10) indicated adequate separation. DNA was visualised under ultra-violet light and the results recorded by photography onto either Polaroid™ type 57 film or Kodak T_{max} 100 film.

9.8.7 Isolation of DNA restriction fragments using Geneclean II® and Mermaid®

The Geneclean II® kit is a commercially available kit from Bio 101 inc. for the isolation of DNA fragments from agarose gels (0.5Kb to 15Kb). Mermaid® is a variation of the Geneclean II® protocol to isolate small DNA fragments (10bp to 200bp). Both systems are based upon the binding affinity of DNA molecules to glass powder (silica) (Vogelstein and Gillespie, 1979). The protocols used were based upon those supplied with the commercial isolation kits.

GENECLEAN II®:

The DNA fragment of interest was excised from a agarose gel, 2.0 volumes of NaI solution were added and incubated at 55°C for five minutes. Glassmilk® suspension was then added (5µl Glassmilk® per 5µg or less DNA) and incubated on ice for five minutes. This glassmilk-DNA complex was collected by centrifugation at 13,000 rpm for five seconds and washed three times in New wash solution. DNA was then eluted from the glassmilk by incubation at 55°C for three minutes into a final volume of 10µl sterile distilled water.

MERMAID®:

The DNA fragment of interest was excised from a 2%(w/v) agarose gel and the gel slice incubated in 1ml 1x MEB buffer at room temperature for fifteen minutes. MEB buffer was then discarded. Three volumes HSBS (High Salt Buffer Solution) and 10µl Glassfog® were added. This suspension was vortexed for fifteen minutes at room temperature, collected by centrifugation at 13,000 rpm for

ten seconds and resuspended in 300µl ethanol-wash buffer. The centrifugation and wash step were repeated and the Glassfog/DNA pellet resuspended in 10µl sterile distilled water. DNA was then eluted by incubation at 55°C for five minutes and Glassfog® removed by centrifugation. The eluted DNA fragment was stored at -20°C prior to use.

9.8.8 'Southern' transfer of DNA (Southern, 1975)

A modification of the protocol of (Southern, 1975), for the analysis of DNA was used.

DNA to be examined by 'Southern' hybridization was digested with suitable restriction endonuclease(s) and separated on an agarose gel (see section 9.8.6). The amounts of DNA loaded were approximately 10µg genomic DNA and 1µg plasmid DNA per lane. For large molecular weight DNA, a depurinating step is necessary for efficient transfer of DNA. DNA was depurinated in the agarose gel by soaking twice, in 0.25N HCl for fifteen minutes. Excess HCl was removed by washing in distilled water. DNA denaturation was in 0.5M NaOH, 1.5M NaCl with gentle shaking for forty minutes, the gel was then washed in distilled water and neutralised in 0.5M Tris-HCl (pH7.4), 1.5M NaCl for forty minutes with gentle shaking, followed by another wash with distilled water.

DNA was then transferred by capillary action as described by (Fourney *et al.*, 1988), using 20x SSC, onto Amersham Hybond-N™ filter. Transferred DNA was cross-linked onto the filter membrane with U.V radiation and filters were stored in Saran-wrap™ at room temperature prior to hybridization.

9.8.9 RNA gel electrophoresis and 'Northern' transfer

RNA samples were analysed by formaldehyde/agarose gel electrophoresis, which provides good size separation and resolution in a simple denaturing electrophoresis system (Leharch *et al.*, 1977). A modified protocol by (Praekelt and Meacock, 1990) was used in the analysis of total cell RNA isolated in this study.

Yeast total cell RNA was isolated using the methods as outlined in section 9.6. RNA samples of upto 10µg were made upto a 5µl volume with sterile distilled water, to which 2µl 5x MOPS, 10µl de-ionised formamide and 3.5µl

formaldehyde (40%(w/v)) were added. Samples were then heat denatured at 65°C for ten minutes, 3.5µl sterile RNA loading buffer was added and this reaction mix was analysed on a 1.5%(w/v) agarose/6%(v/v) formaldehyde gel /1x MOPS gel (Fourney *et al.*, 1988), at a constant voltage (80v) for two to three hours in 1x MOPS buffer. Ethidium bromide (1mg ml^{-1}) was incorporated into the loading buffer (Shelness and Sullivan, 1986), in order to visualise RNA and assess the level of RNA loading. After electrophoresis RNA was transferred overnight by capillary action (Fourney *et al.*, 1988), with 10x SSC onto Amersham Hybond-N™ and cross-linked onto the filter membrane using U.V radiation. Filters were stored at room temperature in Saran-wrap™, prior to hybridization.

9.8.10 'Dot-blot' hybridization

'Dot-blot' hybridization provides a rapid detection of either RNA or DNA in samples, where the direct transfer of RNA or DNA to 'spots' or 'dots' on Hybond-N is made. The spotted filter is then hybridized with a labelled probe. 'Dot-blot' membranes were prepared using a BioRad 96-well Bio-Dot microfiltration unit. The set-up procedure was as follows. One piece of Hybond-N membrane and an equal size of Whatman 3MM paper (cut to the size of the apparatus) were soaked in 10x SSC. The Whatman filter was placed down first, followed by the Hybond-N membrane. The apparatus lid was fitted and latched into place. Each well was rinsed with 100µl 20x SSC and drawn through under vacuum. Marker dye was placed in the outer wells in order to orientate the filter. For an RNA 'dot-blot', 1-2µg RNA in 50µl 20x SSC was added to the wells. For a DNA 'dot-blot' 1-2µg of DNA in 10µl TE buffer was heated to 95°C for five minutes, then a further 40µl 20x SSC was added, prior to loading onto the apparatus. Samples were drawn through the membrane under vacuum and each well was rinsed with 100µl 20x SSC. The nucleic acid was then cross linked to the membrane by UV radiation. Hybridization conditions were the same as those described for both 'Northern' and 'Southern' hybridization.

9.8.11 Preparation of high specific activity probes with ^{32}P α-dCTP based on the method of (Feinberg and Vogelstein, 1983).

Radioactive DNA probes were prepared by random hexadeoxynucleotide priming of DNA fragments, based upon the method of (Feinberg and Vogelstein, 1983).

The following buffers were prepared and stored at -20°C:

Solution A	1.0ml solution O, 18µl ß-mercaptoethanol, 5µl each dNTP except dCTP
Solution B	2M HEPES-NaOH (pH6.6)
Solution C	Hexanucleotides at 90 A_{260} ml^{-1} in TE buffer
OLB 'C' buffer	A mixture of A:B:C in the ratio 10:25:15 volumes.
dNTP's	100mM dATP, dTTP, dCTP and dGTP in distilled water
Solution O	1.25M Tris-HCl (pH8.0), 0.125M $MgCl_2$

The DNA fragment to be used as a 'probe', was separated and isolated on a agarose gel as described in section 9.8.6 and 9.8.7. Approximately 200ng DNA fragment in 14µl sterile distilled water was heat denatured at 100°C for three minutes followed by centrifugation for ten seconds at 13,000 rpm. To the denatured DNA fragment the following were added; 5µl OLB 'C' buffer, 1µl BSA (10mg ml^{-1}), 2.5µl [α-^{32}P] dCTP (10µCi μl^{-1}, ≥3000ci $mmol^{-1}$) (Amersham International) and 1u 'Klenow' fragment (large fragment DNA polymerase I) (BRL Ltd) and incubated at room temperature for between four to twenty hours.

9.8.11.1 Removal of un-incorporated nucleotides from radiolabelled probes

Un-incorporated radionucleotides were removed from the label reaction prior to hybridization in order to reduce non-specific binding/hybridization by column fractionation on a Sephadex® G50 (medium) column (Pharmacia) (Maniatis *et al.*, 1982). The label reaction volume was increased upto 100µl with 1x TE (pH8.0), prior to loading onto the column and the labelled probe DNA was eluted in 100µl fractions with 1x TE (pH8.0). Aliquots containing the radiolabelled probe, were pooled, prior to heat denaturation and addition to the pre-hybridized filters.

9.8.12 DNA/RNA hybridization with radiolabelled probe DNA

Church-Gilbert buffer (Church and Gilbert, 1984) was used for both 'Southern' and 'Northern' hybridization analysis.

Filters prepared (see section 9.8.8, 9.8.9 and 9.8.10) were pre-hybridized in 10ml Church-Gilbert buffer (7%(w/v) SDS, 1mM EDTA, 0.5M $NaHPO_4$ (pH7.4)), at 65°C for between four and twenty hours with constant agitation. Fractionated radio-labelled probe DNA was pooled and heat denatured at 100°C for five minutes, prior to addition to the hybridization reaction. Hybridization was continued overnight (≥18 hours) at 65°C with constant agitation.

After an appropriate hybridization period, filters were washed twice in a solution of 3x SSC, 0.1%(w/v) SDS (at 65°C), for thirty minutes at 65°C with gentle agitation. The filter was blotted dry, wrapped in Saran-wrap™ and exposed at -70°C, with intensifying screens for varying lengths of time dependent upon the level of radioactivity (usually between twelve to seventy-two hours). The film used was Amersham Hyperfilm-MP™.

9.8.13 Polymerase chain reaction

The polymerase chain reaction (PCR) was used in the construction of the disruption cassettes (see chapter seven) and the detection of transcription of the *THI5* open reading frame (see chapter seven). PCR reactions were performed in a 20µl reaction volumes, in 0.5ml Sarstedt tubes with mineral oil overlay to minimise evaporation. Each reaction contained between 200-600ng plasmid DNA, 1x PCR buffer (New England Biolabs), 2mM dNTP mix (dATP, dTTP, dCTP and dGTP), 20pmol of each 5' and 3' oligonucleotide primer and 10mM $MgSO_4$. Two units of Deep $Vent_R$® DNA polymerase (New England Biolabs) was added to each tube. The following cycles were used to amplify a region of 1.0Kb, extension times were varied dependent upon the size of the region to be amplified (a guide to extension times was 1,000 bases per minute). The first denaturation step was 95°C for 5 min, followed by 25 cycles; denaturation: 94°C for 30 seconds, annealing: 54° for 60 seconds; extension 72°C for 60 seconds.

Oligonucleotide primers were engineered to contain desired restriction endonuclease sites as described by (Scharf, 1990). The PCR reaction mix was digested with the appropriate restriction endonucleases and the DNA fragment purified by agarose gel electrophoresis and Geneclean II® extraction, prior to the cloning stage (see appropriate sections).

9.9 Bacteriophage techniques

9.9.1 Lambda vectors and bacterial host strains

A genomic DNA library was constructed in the lambda replacement vector, λEMBL3 (Fischauf *et al.*, 1983), with λEMBL3 *Bam*HI arms and packaging extract, both supplied by Promega.

9.9.2 *In vitro* packaging of ligation mix

In the preparation of a genomic library, partial digested genomic DNA was ligated to λEMBL3 arms and packaged into bacteriophage λ host/vector.

The ligation mix (10µl) was packaged *in vitro* using the Promega Packagene® system. The Packagene® extract was thawed and stored on ice, a $^{1}/_{5}^{th}$ volume of extract was then added to each ligation reaction and mixed by gentle tapping. Packaging was carried out at 22°C for two hours and the reaction volume was increased upto 500µl with phage buffer (20mM Tris-HCl (pH7.4), 100mM NaCl, 10mM $MgSO_4$). 25µl chloroform was added and the packaged phage stored at 4°C, prior to titring and amplification.

9.9.3 Screening/Titration of lambda library

9.9.3.1 Titration of library

After packaging of the ligation mix, the titre of the library was calculated, by infection of plating cells with sterile dilutions of packaged λ library in phage buffer ($1:10^4$ to $1:10^6$). LE392 (see section 9.1) plating cells were prepared by inoculation of a single colony into 50ml LB medium supplemented with maltose to a final concentration of 0.2%(w/v), $MgSO_4$ to 0.1M and incubated at 37°C overnight with constant agitation (Promega, 1991).

Diluted phage (100µl) was added to an equal volume of LE392 plating cells and the absorption of the bacteriophage was allowed for thirty minutes at 37°C. 3ml TB top agar (45°C) was added to the absorbed phage/bacteria and mixed via gentle vortexing. Pre-warmed LB agar plates (37°C) were over-layered with the TB agar and incubated at 37°C overnight. The titre of the library was determined by the number of plaque forming units multiplied by the dilution factor and a factor of ten to give plaque forming units (pfu) per millilitre.

9.9.3.2 Amplification of library

Amplification of the bacteriophage library is necessary for preservation of the number of copies within the library. 200µl plating cells were transfected with the titred package mix containing $2x10^4$ pfu, with absorption at room temperature for twenty minutes. Overlay plates were prepared and incubated at 37°C for between

six to ten hours. Before phage plaques became confluent, 2ml LB broth was used to flood the overlay agar plate and the top agar scraped off with a sterile bent glass pipette. 2ml chloroform was added per 100ml excised top agar and any cell debris removed by centrifugation in sterile conical tubes at 3,000 rpm for ten minutes. The supernatant containing the amplified library was decanted and crystalline NaCl added to a final concentration of 1M. The titre of the library was repeated and the library stored in 1ml aliquots in sterile glass bottles with 50µl chloroform at 4°C or at -70°C with DMSO at a final concentration of 7%(v/v).

9.9.3.2 Screening of library

Selected bacteriophage clones were screened by hybridization to a radiolabelled DNA probe. DNA from plated plaques was partially transferred to nitrocellulose filters for hybridization (Benton and Davis, 1977). Several rounds of plating and screening yield plaque purified clones for further characterization.

Plaques were transferred onto nitrocellulose filters (Sartorious-Membranfilter® 0.45µm) by layering the filter onto the bacterial lawn containing phage plaques. Filters were orientated by marking with needle holes through the filter and media agar. After plaque lift the filters were air-dried for approximately thirty minutes and then sequentially treated on Whatman 3M paper soaked sequentially in; 0.2M NaOH/1.5M NaCl, 2xSSC/0.4M Tris-HCl (pH7.4) and 2xSSC with each treatment for one minute. Transferred bacteriophage DNA was then fixed onto the nitrocellulose filters by baking in a vacuum oven for two hours at 80°C.

Filters were pre-hybridized and probed with radiolabelled probe DNA (as outlined in sections 9.8.11 and 9.8.12). After autoradiography, agar overlay plates were orientated with plaque filter autoradiographs. Plaques which showed a positive hybridization signal were removed from the overlay plate and the bacteriophage eluted into sterile phage buffer (100µl) overnight at 4°C. The screen was then repeated with the eluted bacteriophage and the appropriate radiolabelled probe DNA. A tertiary screen was carried out in order to determine whether the plated phage was homogeneous. After this tertiary screen one plaque was removed, eluted and used to prepare DNA for subsequent sub-cloning into pUC vectors, prior to further analysis.

9.9.4 Lambda DNA isolation by CsCl step gradient (Maniatis *et al.*, 1982)

9.9.4.1 Culture of bacteriophage lambda

E. coli λ sensitive strain, LE392 was grown overnight in 10ml LB broth supplemented with $MgCl_2$ to 10mM and maltose to 0.2%(w/v). To $2x10^8$ pfu (plaque forming units), 0.1ml phage suspension buffer (10mM $MgCl_2$/10mM $CaCl_2$) and 0.1ml overnight saturated culture were incubated at 37°C for twenty minutes without agitation. 100ml pre-warmed LB broth supplemented with $MgCl_2$ to 10mM was inoculated with the above culture and incubated at 37°C, 200 rpm for upto seven hours. Cell growth was monitored by extinction changes at 650nm until a sudden drop was observed, indicating cell lysis. Remaining bacterial cells were lysed by the addition of 0.5ml $CHCl_3$ and incubation for a further fifteen minutes.

9.9.4.2 Purification of lambda bacteriophage

Bacterial DNA and RNA was removed from the bacteriophage lysate by incubation at room temperature for one hour with gentle agitation after the addition of 1mg DNaseI (10mg ml^{-1}) (Sigma) and RNase A (10mg ml^{-1}) (Sigma). Cell debris was removed by centrifugation at 10,000 rpm (4°C) for ten minutes and the lysate clarified through glass wool. Intact phage particles were precipitated overnight at 4°C by the addition of solid PEG6000 to a final concentration of 10%(w/v) and NaCl to a final concentration of 1M. Precipitated phage particles were recovered by centrifugation at 10,000 rpm for ten minutes and resuspended in a $^1/_{50}{}^{th}$ final volume phage suspension buffer (10mM $MgCl_2$/10mM $CaCl_2$) at 4°C. Resuspended bacteriophage particles were clarified by centrifugation at 10,000 rpm (4°C) for ten minutes and stored at 4°C prior to extraction of lambda DNA.

Lambda DNA was purified by centrifugation on CsCl step gradient as outlined in (Maniatis *et al.*, 1982). To a CsCl step gradient of density (ρ)= 1.70, 1.50, 1.45g ml^{-1} in λ Tris- buffer (6mM Tris-HCl (pH8.0), 10mM $MgCl_2$, 100mM NaCl, 0.5mg ml^{-1} gelatine), the lambda bacteriophage suspension was carefully layered onto the top gradient (ρ = 1.45 g ml^{-1}) and the remaining volume of the centrifuge tube made up with λ Tris- buffer. Tubes were balanced to within 0.01g and centrifuged at 35,000 rpm for 120 minutes in a Sorvall® TST41.14 swing-out rotor using 14ml buckets. Centrifuge tubes were removed very carefully, in order to minimise disruption of the step gradient. Intact bacteriophage particles were

visible as a lower blue band and extracted with a needle and syringe. This phage band was dialysed against 1x TE (pH8.0) for one hour at room temperature and extracted three times with phenol/$CHCl_3$/IAA. Phage DNA was precipitated with $^1/_{10}{}^{th}$ volume 3M sodium acetate (pH7.0), 2 volumes 100%(v/v) ethanol and centrifuged at 10,000 rpm for ten minutes. Precipitated DNA was resuspended in an appropriate volume of sterile distilled water and the DNA concentration determined by absorption at 260nm on a Shimadzu spectrophotometer.

9.10 DNA sequencing

9.10.1 Exonuclease III unidirectional 'nested' deletions for sequencing

DNA clone fragments in pUC based vectors, were sequenced by the construction of nested deletions based upon exonuclease III digestion (Henikoff, 1987). Plasmid DNA (5µg) containing the gene of interest, was digested with appropriate restriction enzymes to generate the necessary 3' and 5' termini for exonuclease digestion (Promega, 1989; Promega, 1990). Nested deletions were made using a modified protocol based upon (Ausebel, 1991), where the following modifications were made to that protocol.

After restriction endonuclease digestion, the reaction mix was extracted once with phenol/$CHCl_3$/IAA, followed by ethanol precipitation. The digested plasmid DNA was resuspended in 1x ExoIII buffer (15mM Tris-HCl (pH8.0), 0.66mM $MgCl_2$) to a final concentration of 0.1µg ml^{-1}. This reaction mix was pre-incubated at 37°C for thirty seconds prior to the addition of 150u exonuclease III (BRL Ltd), followed by exonuclease III digestion at 37°C over a ten minute period. Aliquots (6µl) of this digestion mix were taken every minute and flash frozen to -20°C.

The exonuclease was inactivated by incubation at 70°C for ten minutes and the reaction volume increased to 25µl with sterile distilled water. Exonuclease VII was used to remove the single stranded DNA produced by the action of exonuclease III (Ozkaynak and Putney, 1987). To the above reaction mix, 2µl 10x ExoVII buffer (100mM Tris-HCl (pH7.5), 100mM EDTA, 300mM KCl), 2u exonuclease VII (BRL Ltd) were added and the reaction mix incubated at 37°C for one hour. The reaction volume was increased to a total of 50µl with sterile distilled water and extracted once, with phenol/$CHCl_3$/IAA, followed by ethanol precipitation.

The precipitated DNA was then resuspended in 10µl 1x ligation buffer (80mM Tris-HCl (pH7.5), 30mM DTT, 20mM $MgCl_2$) and 2u large DNA polymerase 'Klenow' fragment added. The reaction mix was pre-incubated at 37°C for two minutes, 4µl dNTP (0.25mM each dNTP) added and the reaction mix incubated for a further ten minutes at 37°C. A further 2µl 10x ligation buffer, 3µl 10mM ATP, 8u T4 DNA ligase (Pharmacia) were then added and the reaction volume increased to a total of 20µl. The ligation reaction was carried out at 15°C, overnight (approximately eighteen hours) and the entire ligation mix (a total volume of 20µl) used to transform competent *E. coli.*

Correct deletions within the plasmid were screened by appropriate restriction endonuclease digestion of mini-prep plasmid DNA. Plasmid clones showing an sequential size loss of 200-300bp were used in subsequent DNA sequence analysis.

9.10.2 Sequencing reaction with T7 DNA polymerase

Sequencing reactions were performed with a modified T7 DNA sequencing kit™ (supplied by Pharmacia), with [α ^{35}S]-dATP obtained from Amersham International. The following modifications were made to the supplied T7 DNA sequencing protocol.

Double stranded DNA sequencing reactions were performed using plasmid DNA prepared either by CsCl density gradient or 'midi-prep' plasmid DNA (see section 9.5). Plasmid DNA (2µg) was denatured using 2M NaOH (as outlined in supplied protocol), ethanol precipitated and resuspended in a final volume of 9µl sterile distilled water.

To 9µl resuspended denatured DNA, 13ng primer (16-mer) was annealed at 37°C for twenty minutes and further incubated at room temperature for twenty minutes in the supplied annealing buffer. Labelling and extension reactions were as outlined in Pharmacia protocols. The completed reaction mixes were stored at -20°C, prior to analysis on a denaturing polyacrylamide gel.

9.10.3 Resolution, gel fixation and exposure

Sequencing reactions were analysed on a 9% denaturing polyacrylamide gel. The gel was prepared using Accugel™ acrylamide:bis-acrylamide (19:1(v/v)), with urea (Sigma) added to a concentration of 7M. Polymerization of the

polyacrylamide gel was as outlined in (Maniatis *et al.*, 1982) with TEMED (Sigma) and 10%(w/v) ammonium sulphate. The gel (30cm x 40cm x 0.3mm) was poured and allowed to polymerize for approximately one hour prior to loading onto a BRL S2 sequencing gel electrophoresis apparatus. The sequencing gel was then pre-run for one hour at a constant 1800v, to ensure the uniform heating of the gel.

After pre-running of the gel, 2.5µl of the DNA sequence reaction mix was heat denatured at 100°C for three minutes prior to loading onto the polyacrylamide gel. The gel was loaded twice, once when the bromophenol blue marker dye in the sample buffer had reached the bottom of the gel. At this point a further 2.5µl sample was then loaded and the run continued until the bromophenol blue marker again reached the bottom of the gel, thus allowing the resolution of between 250-350bp of DNA sequence.

After electrophoresis the polyacrylamide gel was fixed to remove the urea within the polyacrylamide sequence gel with 10%(v/v) glacial acetic acid, 10%(v/v) methanol, for fifteen minutes. The gel was wash with distilled water, blotted dry onto a Whatman 3M paper backing, covered with Saran-wrap® and dried under vacuum at 80°C until dehydrated. The Saran-wrap® was removed and the gel exposed at room temperature onto Amersham Hyperfilm-MP™, where exposure times at room temperature were dependent upon the activity of the gel obtained.

9.10.4 Oligonucleotide primers and primer walking

Plasmids containing 'nested' deletions were sequenced using the universal M13 forward and reverse primers obtained from New England Biolabs, which correspond to regions adjacent to the multiple cloning site within the pUC vectors used in the initial cloning. The primers supplied by New England Biolabs were designated;

NEB#1233 (5' AGCGGATAACAATTTCACACAGGA 3')
NEB#1224 (5' CGCCAGGGTTTTCCCAGTCACGAC 3')

Extension of DNA sequence by primer walking (Strauss *et al.*, 1986) was used to extend sequence data between deletion clones of greater than 350-400 bp separation. Primers were designed with at least a 50% GC content, especially in

the 3' region, with the average length of each primer between 16- and 18-mers. The primer was designed to be approximately between 60-80bp from the terminal end of the previous set of sequence reactions. Primers were obtained from the Department of Biochemistry, Leicester University and J. Kite, Nottingham University and were precipitated using sodium acetate/ethanol. The primer was resuspended in 50µl sterile distilled water and oligonucleotide concentration determined by measurement of absorption at 260nm. Primers were then diluted in sterile distilled water to a working concentration of 0.0044µg μl^{-1} (equivalent to primer concentration used in the T7 DNA polymerase sequencing kit^{TM}(Pharmacia)).

9.11 ^{35}S Methionine *in vivo* labelling of proteins

9.11.1 ^{35}S methionine *in vivo* labelling procedure after (Hames, 1981)

To a 0.5ml sample of yeast cell suspension, 1µl ^{35}S-methionine (37 TBq $mmol^{-1}$, Amersham international) was added, incubated at 30°C for ten minutes and cooled on ice, prior to centrifugation at 13,000 rpm for one minute. The supernatant was discarded as radioactive waste and the cell suspension washed, twice with 1ml ice-cold sterile distilled water.

9.11.2 Yeast protein extraction after (Ausubel *et al.*, 1989)

The cell pellet was resuspended in 100µl cold lysis buffer (50mM Tris-HCl (pH7.2), 1%(w/v) deoxycholate, 1%(v/v) Triton x100, 0.1%(w/v) SDS +2mM PMSF prior to use)). An equal volume of glass beads (2µM diameter) was then added and vortexed five times, for a thirty second duration, with cooling on ice, between each vortex step. To the cell lysate an equal volume of SDS/loading buffer (2x) was added and the samples stored at -20°C, prior to analysis by SDS/polyacrylamide gel electrophoresis.

9.11.3 Protein gel electrophoresis (Hames, 1981)

In vivo labelled proteins were analysed by SDS/polyacrylamide gel electrophoresis (Ausubel *et al.*, 1989; Hames, 1981). A 10.5%(v/v) non-denaturing polyacrylamide gel was prepared in a Biorad® protein II dual slab cell apparatus. The gel was run at 100v until the loading dye reached the bottom of the gel. A Protein marker (Amersham [^{14}C] methylated Rainbow^{TM} protein molecular weight marker MW 14,300-200,000) was included on the gel in order to size the labelled proteins. After electrophoresis, the gel was fixed in 10%(v/v) acetic acid, 30%(v/v) methanol for twenty minutes. Excess gel fix was removed with distilled

water and the gel blotted onto a Whatman 3M paper backing. The gel was covered with Saran-wrap® and dried under vacuum at 80°C. After drying the Saran-wrap® was removed and autoradiography of the gel was carried out at room temperature onto Amersham Hyperfilm-MP™.

9.12 Chromosome analysis by CHEF gel electrophoresis

9.12.1 Preparation of CHEF plugs (Gerring *et al.*, 1991).

An 50ml overnight culture of the *Saccharomyces cerevisiae* strain, YPH149 (Carle, 1986; Gerring *et al.*, 1991)) was grown to a density of $1.0x10^7$ cells ml^{-1}. Yeast cells were collected by centrifugation at 3,500 rpm for five minutes (20°C) and the cell pellet resuspend in 2.5ml sphaeroplast buffer (0.9M sorbitol, 50mM $NaPO_4$ (pH7.5), 14mM ß-mercaptoethanol). 100µl yeast lytic enzyme (10µg ml^{-1} in distilled water) was added and the cell suspension incubated at 30°C with gentle agitation for fifteen to thirty minutes, with sphaeroplast formation checked by examination of yeast cells. Sphaeroplasts were embedded in an equal volume 1%(w/v) LGT (SeaKem® low-gelling temperature) agarose in 1x sphaeroplast buffer in a gel former and cooled to 0°C on ice for approximately fifteen minutes. Agarose plugs were then washed twice in SET buffer (1%(w/v) sarcoysl, 450mM EDTA (pH9.0), 10mM Tris-HCl (pH8.0)). SET buffer was removed and replaced with an equal volume SET buffer plus proteinase K (1mg ml^{-1}) and incubated at 37°C for two hours, without agitation. Proteinase K was removed by washing twice, in 0.5M EDTA (pH9.0) and agarose plugs were stored at 4°C in 0.5M EDTA (pH9.0), prior to use.

CHEF plugs were analysed on a 1.0%(w/v) agarose gel in 0.5x TBE, with approximately $^1/_2$ an agarose plug loaded per lane. The gel was run in a Biorad CHEF DR-II® electrophoresis apparatus with the gel immersed under 0.5x TBE at 14°C, with constant buffer circulation. Chromosomes were resolved at 200v by a pulse time of 60 seconds for fifteen hours, followed by a pulse time of 90 seconds for eight hours, with a total run time of twenty three hours.

9.12.2 Chromosome transfer and hybridization conditions

The CHEF gel was stained with ethidium bromide (0.5µg ml^{-1}) for thirty minutes and destained with 10mM $MgSO_4$ for between one and two hours. The gel obtained was photographed (a scale was included to allow identification of

chromosomal location by comparison of migration distance) and transferred onto a support membrane (section 9.8.8), prior to hybridization (section 9.8.11 and 9.8.12).

9.13 Enzyme assay

Enzyme assays were used to determine the carbon levels during growth in fermenter grown cultures and was to determine whether gene expression was correlated to carbon source utilisation (see chapter two and three). All absorption measurements were made on a Phillips Unicam spectrophotometer.

9.13.1 Glucose

Glucose concentration was determined using a glucose oxidase based, assay kit obtained from Sigma (Procedure No. 510). Absorption measurements were made on a Phillips Unicam spectrophotometer at 425nm. Three successive replicates of each sample were taken. The average of these samples was then taken as representing the concentration of glucose present.

9.13.2 Raffinose

The concentration of raffinose within the media was determined using a linked enzyme assay of α-galactosidase and galactose dehydrogenase as outlined by (Bergmeyer, Bernt and Gutmann, 1974). Optical density measurements were taken at 340nm. An average value of three samples was taken to represent the level of raffinose present in the media sample.

9.13.3 Lactate

D-lactate levels were assayed for using D-lactate dehydrogenase after the method of (Gawehn and Bergmeyer, 1974). Replicates of three samples were determined and the average result was taken to represent the level of D-lactate within the sample. Measurement of extinction changes were taken at 340nm.

9.13.4 Ethanol

Ethanol concentration within media samples was determined using a Sigma 332-A test kit. Absorption measurements were made at 340nm. The test was duplicated three times and the average of these results was taken to be the level of ethanol present within the sample. The test was based upon the alcohol dehydrogenase/NAD system of (Bernt and Gutmann, 1974).

APPENDICES

The proceeding appendices contain the following:

Appendix I: Oligonucleotide primer sequences
Appendix II: Restriction endonuclease plasmid maps
Appendix III: DNA sequence RH3 and inferred amino acid ORF
Appendix IV: DNA sequence RH4/11 and inferred amino acid ORF

APPENDIX I:

Oligonucleotide primer sequences

The following primers were used in the sequencing protocols for clones pRH3, pRH4, pRH8, pRH11, ESP30 and ESP65 clones. The individual primers were synthesized by J. Kite, Department of Genetics, Nottingham University and Department of Biochemistry, Leicester University.

Primer: 5'						3'
1/1	ATT	GTG	TAT	GAG	AAG	TGC
2/1	TAA	TTG	TAT	TGA	TAC	TGC
2/2	GAG	TTA	TGG	ATA	GTT	TGC
5/1	CAA	GCG	AAA	CAT	TGA	GGC
5/3	CGG	TAA	GAT	TGA	TGC	TGG
5/4	CCG	ACT	ACG	TTC	TAG	CC
5/5	CTC	ACA	TCG	ATG	TCT	GCC
5/7	TAC	CAA	AGA	TGT	TAC	GC
5/9	AAG	CTT	GTT	ATA	CAT	GC
RH1	CAT	ATT	TTG	GCG	ATG	C
RH2	GCA	AAA	CAA	CGC	ACG	G
RH3	CAC	GTG	CAC	AAA	TTC	G
RH4	ATG	TTC	GCT	CTA	AGG	C
RH5	TTC	GAT	ACT	TGG	AGG	C
RH6	CTA	CAG	AGT	TAG	GAC	G
RH7	CAG	AAG	CTT	GGA	TCG	G
RH10	GTC	GAC	CTT	ACC	AGA	TCC
RH11	GAA	CAA	AAG	AAT	GGA	C
RH13	TAT	GCA	CTA	ACG	CGC	C
C1	TTC	TTT	ACA	TCT	GCA	ACG
C2	AAA	TGC	AGA	CAG	GAA	GG
C3	AAT	TTA	ATA	CTC	AAA	GCC
C4	ACC	ATG	AGA	TGT	AGC	G
C5	CCT	TAT	TCA	TAG	AGC	CC
C6	CAG	ATG	AGG	ATA	GGG	
C7	ATT	CGG	TTA	CTA	CGA	C
C8	GAA	TTG	ATT	GTC	ATG	C
C9	CAT	AGT	TCA	TGA	GCC	G
C10	CAC	ACC	TAG	GGA	TGC	C
C11	CCA	GAA	TTA	GCG	GTG	
C12	CGA	GGC	TTG	AAG	TCG	
C13	CAA	TCT	TTG	AGC	TTC	C
ESP1	CCG	ACC	CTG	TGA	AGG	C
ESP2	GGA	AGA	AGG	TTA	CCG	G
F1	CCC	GAA	TTC	CCG	GGA	TGT CTA ACA AGA TCA
F2	CCC	GGA	TCC	TCA	CTG	GGA AAC CAC GGG CC
F3	CCC	AAG	CTT	CCC	GGG	AAT TCT GGC TTG AAA
	GTA	CCT	TCC			
F4	CCC	GGA	TCC	GGT	TAC	GGT AAG AGA TTA GCC
TP1	CAG	CCT	TCC	TCT	ACG	AC
TP2 (VI)	CAA	GGG	AAA	AGA	AGC	TG
TP3 (XIV)	ATA	AAC	GAA	GTT	CAT	TT

M13/pUC reverse sequencing primer (-24)
NEB1201 AAC AGC TAT GAC CAT G

M13/pUC sequencing primer (-20)
NEB1211 GTA AAA CGA CGG CCA GT

M13/pUC reverse sequencing primer (-48)
NEB1233 AGC GGA TAA CAA TTT CAC ACA GGA

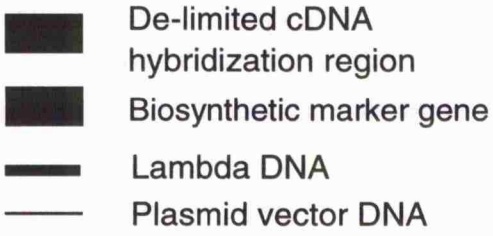
M13/pUC sequencing primer (-47)
NEB1224 CGC CAG GGT TTT CCC AGT CAC GAC

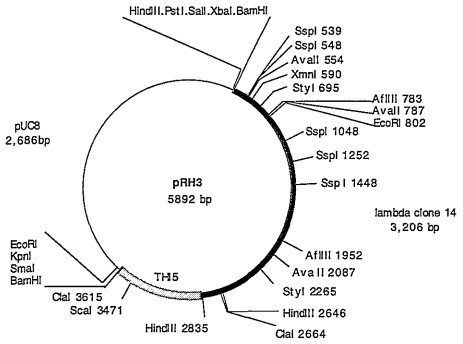
Appendix II

Restriction endonuclease plasmid maps

The following plasmids were constructed and used in the course of this study. Plasmid maps indicating the important restriction endonuclease sites are shown on each plasmid. The position of each site was mapped by sized migration of fragments by agarose gel electrophoresis of individual restriction fragments compared to a known DNA size standard. Various plasmid vectors were used in this study and are not shown, however, the vector is indicated for each plasmid that was constructed. Estimated size of the plasmids is shown for each plasmid and the following key indicates the nomenclature used within the plasmid drawings.

KEY:

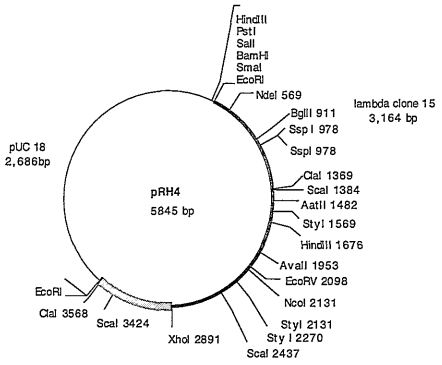




Plasmid name: pRH3

Plasmid size: 5892 bp

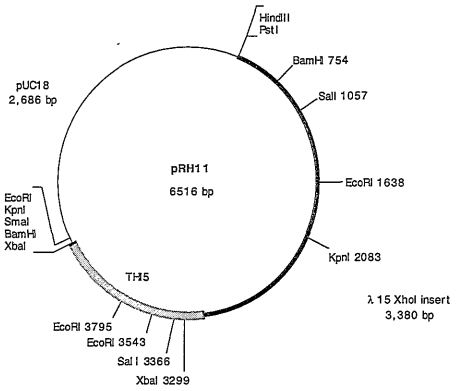
Comments/ References: pRH3 contains the lambda clone 14 BamHI (3,206 bp) fragment. The lambda insert does not contain the following restriction enzyme sites: AatII, ApaI, AvaI, BglI, EcoRV, KpnI, NdeI, NruI, SmaI, SstI, XhoI and XmaI.



Plasmid name: pRH4

Plasmid size: 5845bp

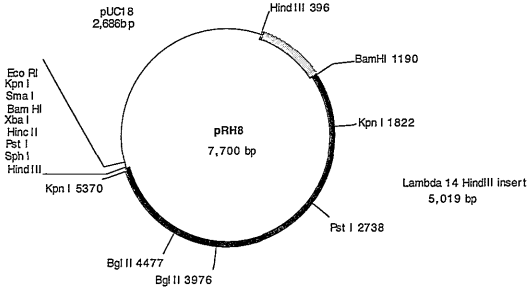
Comments/ References: pRH4 contains lambda 15 EcoRI DNA fragment (3,164bp) pRH4 lambda insert does not contain the following restriction enzyme sites: AflIII, ApaI, BclI, BglI, HpaI, KpnI, NruI, SphI, SstI, XbaI and XmnI.



Plasmid name: pRH11

Plasmid size: 6516 bp

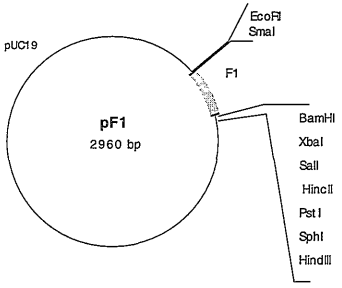
Comments/ References: pRH11 contains λ15 XhoI fragment (3,830 bp) into pUC18 SalI site. pRH11 does not contain the following restriction enzyme sites: HindII, PstI and SmaI.



Plasmid name: pRH8

Plasmid size: 7,700 bp

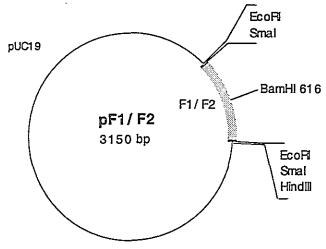
Comments/ References: Lambda 14 HindIII fragment (5,019 bp) cloned into pUC18 on HindIII . Lambda 14 does not contain the following sites: EcoRI, AvaI, SmaI, SphI and XbaI.



Plasmid name: pF1

Plasmid size: 2960 bp

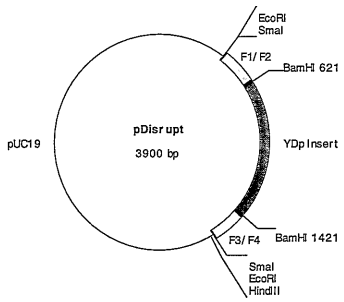
Comments/ References: PCR generated TH5 (chrVI) ORF 5' region (232nt) cloned into pUC19 on enigneered *EcoRI* and *BamHI* restriction endonuclease sites. Oligonucleotide primers F1 and F2 used to generate the F1 fragment.



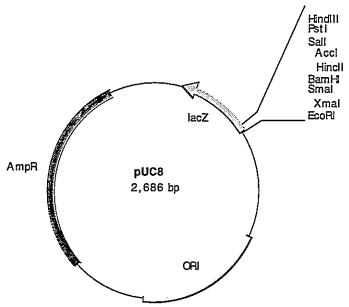
Plasmid name: pF1/F2

Plasmid size: 3150 bp

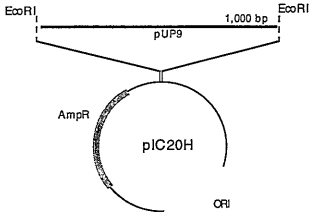
Comments/ References: PCR fragment F2 corresponding to the 3' region of the *TH5* (chrVI) ORF, isolated from the plasmid clone pRH11 using oligonucleotide primers F3 and F4. Fragment F2 was subcloned into plasmid F1 on engineered *HindII* and *BamHI* restriction endonuclease sites. A unique *BamHI* site was formed, flanked by the 5' and 3' DNA fragments of the *TH5* ORF.

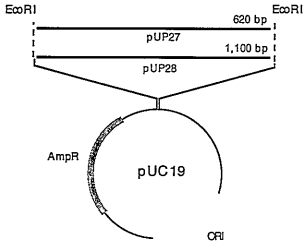


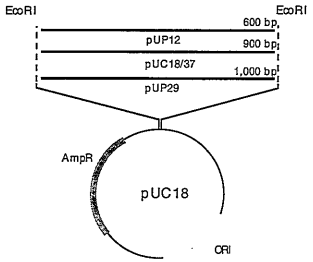
Plasmid name: pDisrupt
Plasmid size: 3900 bp
Comments/ References: pDisrupt series of plasmids, contain TH15 (chr VI) ORF 5' and 3' regions generated by PCR from plasmid clone pRH11. Engineered BamHI restriction site was used to insert yeast biosynthetic marker genes (from YDp plasmid series (see text for details) to form the disruption cartridges.



Plasmid name: pUC8
Plasmid size: 2,686 bp
Comments/ References: Vieira, J. and Messing, J. (1982). Gene. 19, 259.







Key:

Plasmid name	Insert cDNA	Size (bp)
pUP9	ESP35	900 bp
pUP12	HSP16	600 bp
pUP27	ESP30	600 bp
pUP28	ESP65	1,100 bp
pUP29	HXK1	1,000 bp
pUC18/37	HSP26	600 bp

Appendix III

DNA sequence pRH3 (λ^{14} 3.2 Kb *Bam*HI fragment)
From: 1 to: 3211
EMBL Accession number: X83226 (A. Goffeau)

```
          _BamHI_
          GGATCCAAATCGATGAATTGACCAAGCACTACGGTATGAAGCCAGAAGACTACACCGCAG
        1 ---------+---------+---------+---------+---------+---------+ 60
a                                                                       -
b                                                                       -
c           I  Q  I  D  E  L  T  K  H  Y  G  M  K  P  E  D  Y  T  A  V -

          TCAGATGTGGTATGAATGTCGCCAAGTACATCATCGAAGGTAAGATTGATGCTGGTATTG
       61 ---------+---------+---------+---------+---------+---------+ 120
a                                                                       -
b                                                                       -
c           R  C  G  M  N  V  A  K  Y  I  I  E  G  K  I  D  A  G  I  G -

          GTATCGAATGTATGCAACAAGTCGAATTGGAAGAGTACTTGGCTAAGCAAGGCAGACCAG
      121 ---------+---------+---------+---------+---------+---------+ 180
a                                                                       -
b                                                                       -
c           I  E  C  M  Q  Q  V  E  L  E  E  Y  L  A  K  Q  G  R  P  A -

          CTTCTGATGCTAAGATGTTGAGAATTGACAAGTTGGCTTGCTTGGGTTGCTGTTGCTTCT
      181 ---------+---------+---------+---------+---------+---------+ 240
a                                                                       -
b                                                                       -
c           S  D  A  K  M  L  R  I  D  K  L  A  C  L  G  C  C  C  F  C -

          GTACCGTTCTTTACATCTGCAACGATGAATTTTTGAAGAAGAACCCTGAAAAGGTCAGAA
      241 ---------+---------+---------+---------+---------+---------+ 300
a                                                                       -
b                                                                       -
c           T  V  L  Y  I  C  N  D  E  F  L  K  K  N  P  E  K  V  R  K -

          AGTTCTTGAAAGCCATCAAGAAGGCAACCGACTACGTTCTAGCCGACCCTGTGAAGGCTT
      301 ---------+---------+---------+---------+---------+---------+ 360
a                                                                       -
b                                                                       -
c           F  L  K  A  I  K  K  A  T  D  Y  V  L  A  D  P  V  K  A  W -

          GGAAAGAATACATCGACTTCAAGCCTCGATTGAACAACGATCTATCTTACAAGCAATACC
      361 ---------+---------+---------+---------+---------+---------+ 420
a                                                                       -
b                                                                       -
c           K  E  Y  I  D  F  K  P  R  L  N  N  D  L  S  Y  K  Q  Y  Q -

          AAAGATGTTACGCTTACTTCTCTTCATCTTTGTACAATGTTCACCGTGACTGGAAGAAGG
      421 ---------+---------+---------+---------+---------+---------+ 480
a                                                                       -
b                                                                       -
c           R  C  Y  A  Y  F  S  S  S  L  Y  N  V  H  R  D  W  K  K  V -

          TTACCGGTTACGGTAAGAGATTAGCCATCTTGCCACCAGACTATGTCTCGAACTACACTA
      481 ---------+---------+---------+---------+---------+---------+ 540
a                                                                       -
b                                                                       -
c           T  G  Y  G  K  R  L  A  I  L  P  P  D  Y  V  S  N  Y  T  N -
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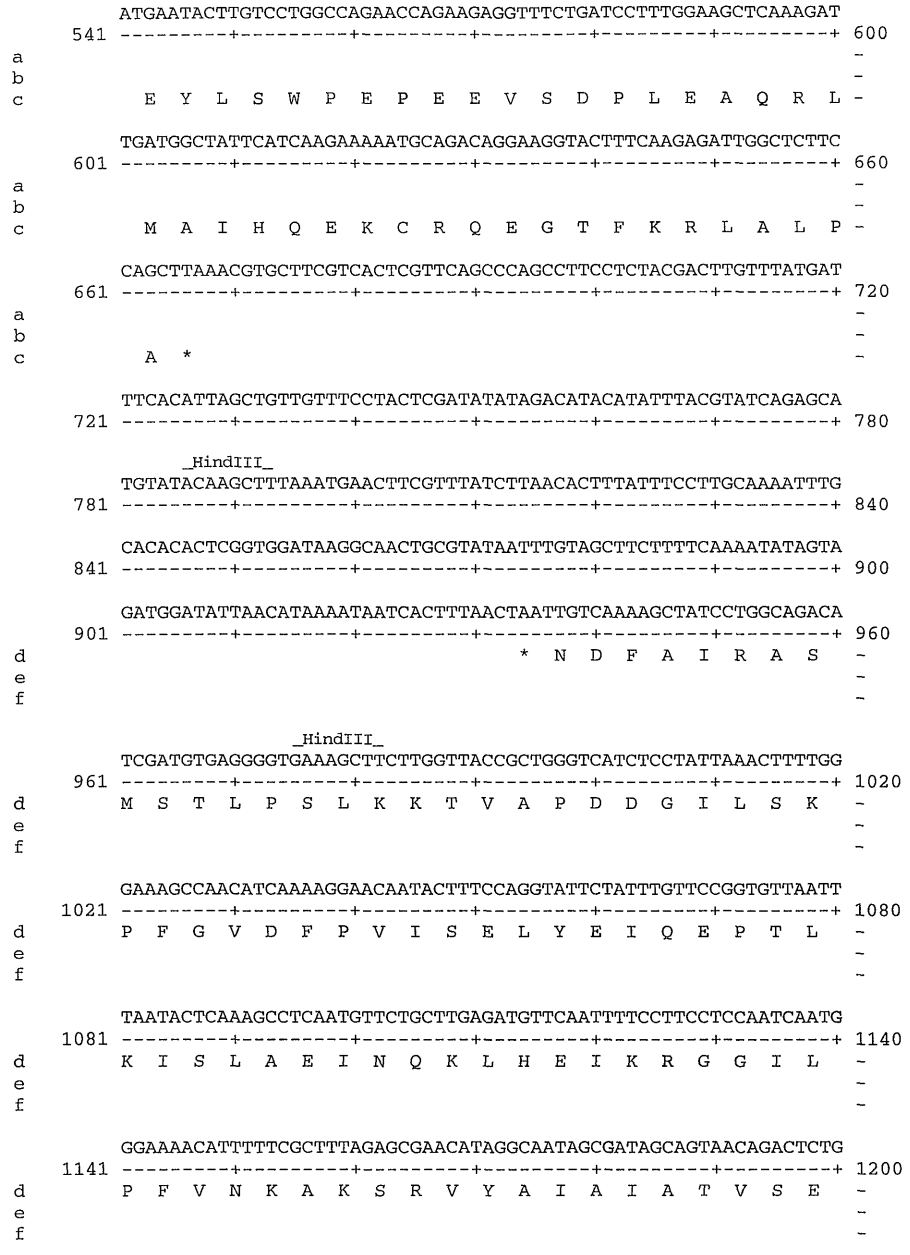
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a -
b -
c E Y L S W P E P E E V S D P L E A Q R L -
TGATGGCTATTCATCAAGAAAAATGCAGACAGGAAGGTACTTTCAAGAGATTGGCTCTTC
601 ---------+---------+---------+---------+---------+---------+ 660
a -
b -
c M A I H Q E K C R Q E G T F K R L A L P -
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661 ---------+---------+---------+---------+---------+---------+ 720
a -
b -
c A * -
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721 ---------+---------+---------+---------+---------+---------+ 780
HindIII
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901 ---------+---------+---------+---------+---------+---------+ 960
d * N D F A I R A S -
e -
f -
HindIII
TCGATGTGAGGGGTGAAAGCTTCTTGGTTACCGCTGGGTCATCTCCTATTAAACTTTTGG
961 ---------+---------+---------+---------+---------+---------+ 1020
d M S T L P S L K K T V A P D D G I L S K -
e -
f -
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1021 ---------+---------+---------+---------+---------+---------+ 1080
d P F G V D F P V I S E L Y E I Q E P T L -
e -
f -
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d K I S L A E I N Q K L H E I K R G G I L -
e -
f -
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e -
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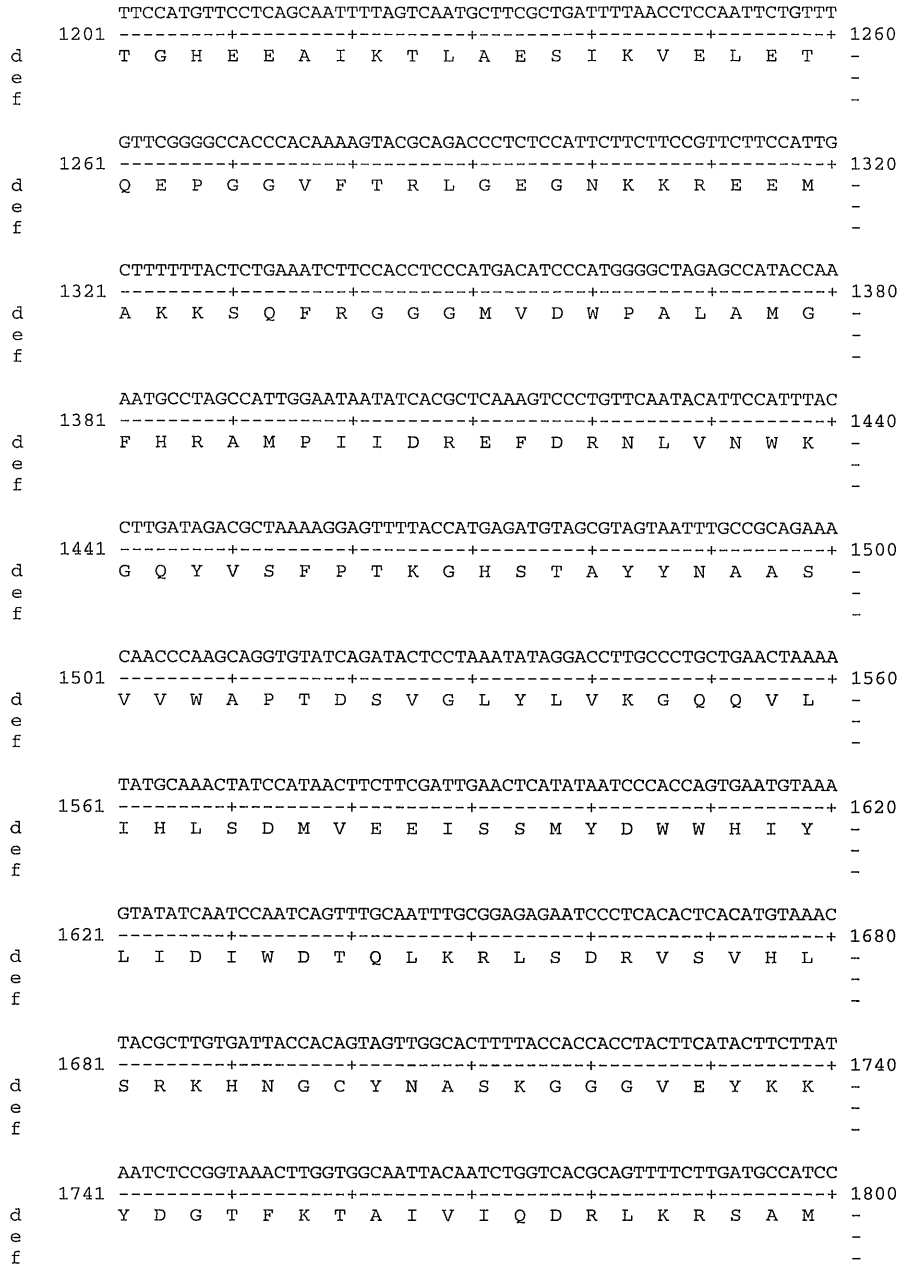
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1261 1320
d Q E P G G V F T R L G E G N K K R E E M -
e -
f -
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1321 1380
d A K K S Q F R G G G M V D W P A L A M G -
e -
f -
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1381 1440
d F H R A M P I I D R E F D R N L V N W K -
e -
f -
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e -
f -
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d V V W A P T D S V G L Y L V K G Q Q V L -
e -
f -
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1561 1620
d I H L S D M V E E I S S M Y D W W H I Y -
e -
f -
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1621 1680
d L I D I W D T Q L K R L S D R V S V H L -
e -
f -
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d S R K H N G C Y N A S K G G G V E Y K K -
e -
f -
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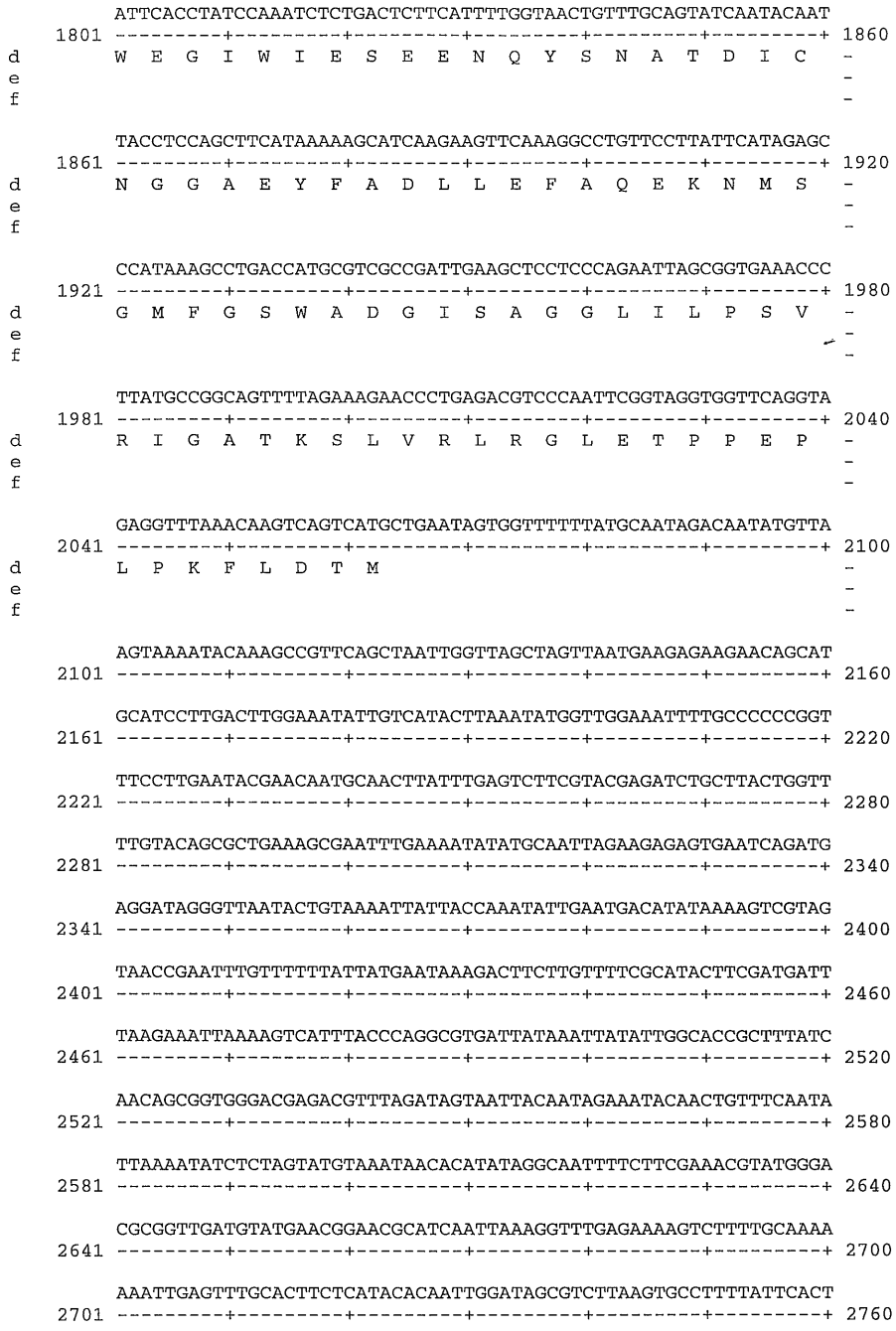
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f -
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1861 1920
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e -
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1981 2040
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e -
f -
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2041 2100
d L P K F L D T M -
e -
f -
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2161 2220
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2221 2280
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2281 2340
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2521 2580
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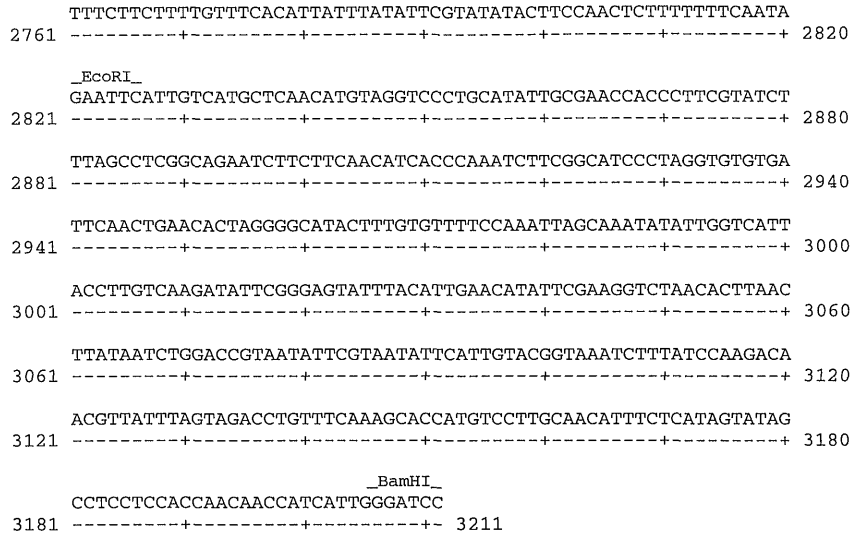
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3121 ----------+----------+----------+----------+----------+----------+ 3180
BamHI
CCTCCTCCACCAACAACCATCATTGGGATCC
3181 ----------+----------+----------+- 3211

Appendix IV

DNA sequence pRH4/11 (λ^{15} *Eco*RI and *Xho*I fragments,respectively)
From: 1 to 4182
EMBL Accession Number: SCTHI5GEN (Z48220)

```
       GTAGAACTAGCGATGCTCACACAACGCTAATTTGTTTCCTTCGAAGGGGCAGTCGGCTAT
     1 ---------+---------+---------+---------+---------+---------+ 60

       TGTAGTTTCTATATATTATGTAGCACAACATGGAAACCATATTCAGCCAGTTTGTATATA
    61 ---------+---------+---------+---------+---------+---------+ 120

       TGAGAATTAAACGTTAAAACTTTTTCATCCTATCAGTCTTTCACATTGGCCTTGCCTAAA
   121 ---------+---------+---------+---------+---------+---------+ 180

       GGGCACAATCGTTCACGTATATACATATGTATGTTTACGTATATATATATGTGTTTACAA
   181 ---------+---------+---------+---------+---------+---------+ 240

       ATATGCATGTATATTCTTTTCGAGGAAGATTATTATATGCTATTAGGCGATGAGTCGGCT
   241 ---------+---------+---------+---------+---------+---------+ 300

       TAATTTTTTAGGGGGACGAGATTGGCGCGTTAGTGCATAAAGACAGCAAATCACCTGGAG
   301 ---------+---------+---------+---------+---------+---------+ 360

       GTGACAACATTAATGCATTTATCGGTTTTTGGCTATGATGCAATGAGTACAGTTCCAATT
   361 ---------+---------+---------+---------+---------+---------+ 420

       GAACGGCTCATGAACTATGAAATATACGAATTATTCAACTAGCAGAAGGCTTGAAATATT
   421 ---------+---------+---------+---------+---------+---------+ 480

       ACTTATTCAGATTATGAAAATGGTGCTGTTATAGCAAAATAAGCAACATCAATCTCTTGC
   481 ---------+---------+---------+---------+---------+---------+ 540

       GACGACAGCAAACAAAACATATAAAAACCTCGTATTATCATCTTCATGAACAATATTCTT
   541 ---------+---------+---------+---------+---------+---------+ 600

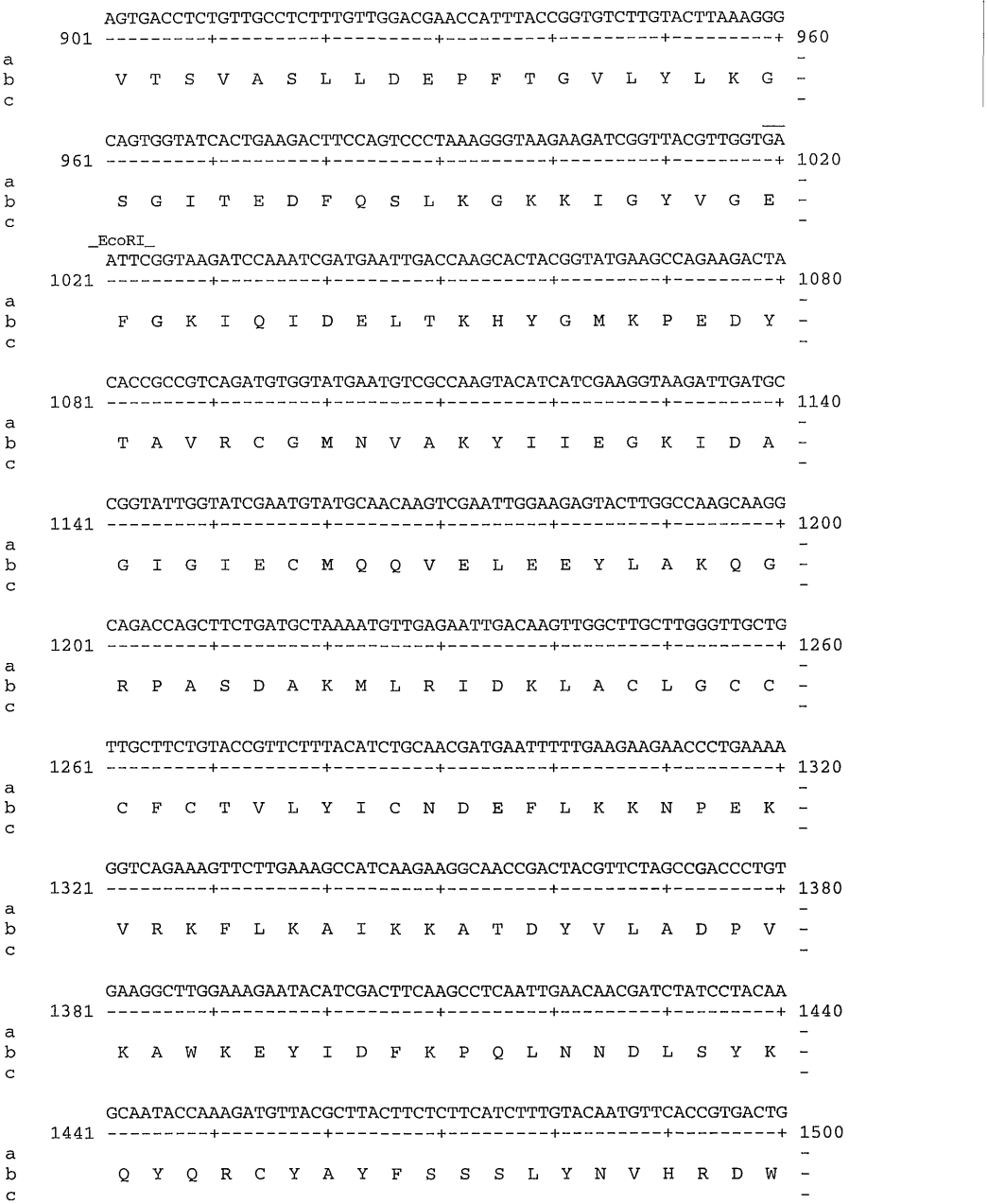
       GTCCATTCTTTTGTTCTTTTACCTTTTCAATTTTATAAAAATACCACAACAACAATAAAC
   601 ---------+---------+---------+---------+---------+---------+ 660

       ACACTTCCAAGCTATGTCTACAGACAAGATCACATTTTTGTTGAACTGGCAACCAACCCC
   661 ---------+---------+---------+---------+---------+---------+ 720
a                                                                    -
b                  M  S  T  D  K  I  T  F  L  L  N  W  Q  P  T  P  -
c                                                                    -

       ATACCATATTCCAATTTTCTTGGCTCAAACCAAAGGTTACTTCAAGGAGCAAGGTCTAGA
   721 ---------+---------+---------+---------+---------+---------+ 780
a                                                                    -
b        Y  H  I  P  I  F  L  A  Q  T  K  G  Y  F  K  E  Q  G  L  D  -
c                                                                    -

       CATGGCCATCCTAGAACCAACCAATCCTTCCGATGTCACTGAGTTAATTGGATCTGGTAA
   781 ---------+---------+---------+---------+---------+---------+ 840
a                                                                    -
b        M  A  I  L  E  P  T  N  P  S  D  V  T  E  L  I  G  S  G  K  -
c                                                                    -

       GGTCGACATGGGTTTGAAAGCCATGATCCACACCTTGGCTGCCAAGGCCCGTGGTTTCCC
   841 ---------+---------+---------+---------+---------+---------+ 900
a                                                                    -
b        V  D  M  G  L  K  A  M  I  H  T  L  A  A  K  A  R  G  F  P  -
c                                                                    -
```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961 1020
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EcoRI
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1021 1080
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1081 1140
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1141 1200
G I G I E C M Q Q V E L E E Y L A K Q G
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1201 1260
R P A S D A K M L R I D K L A C L G C C
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1261 1320
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1321 1380
V R K F L K A I K K A T D Y V L A D P V
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1381 1440
K A W K E Y I D F K P Q L N N D L S Y K
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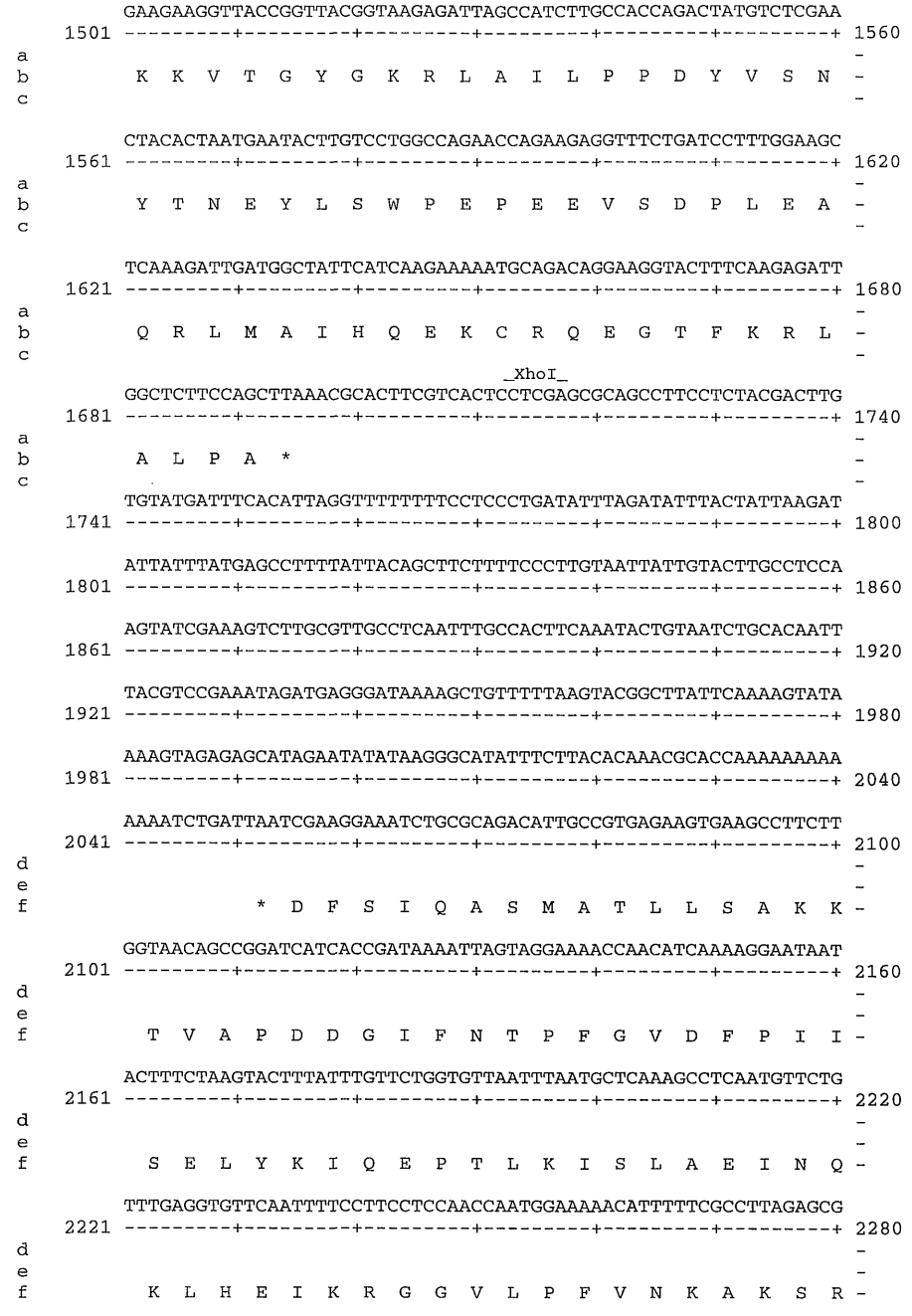
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Y T N E Y L S W P E P E E V S D P L E A
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1621 1680
Q R L M A I H Q E K C R Q E G T F K R L
XhoI
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1681 1740
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2161 2220
S E L Y K I Q E P T L K I S L A E I N Q
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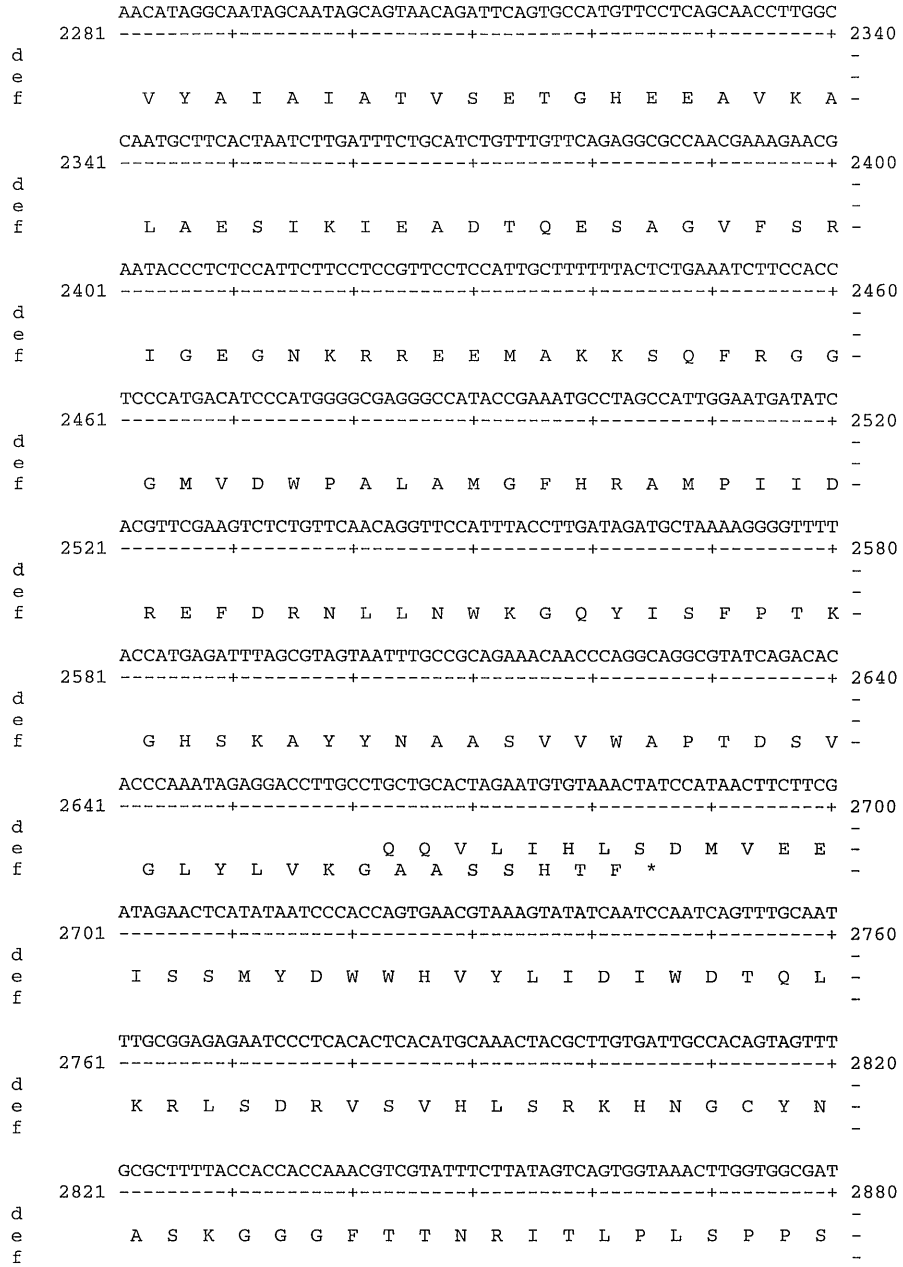
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e
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2341 2400
d
e
f L A E S I K I E A D T Q E S A G V F S R -
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2401 2460
d
e
f I G E G N K R R E E M A K K S Q F R G G -
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2461 2520
d
e
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2521 2580
d
e
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d
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d
e K R L S D R V S V H L S R K H N G C Y N -
f
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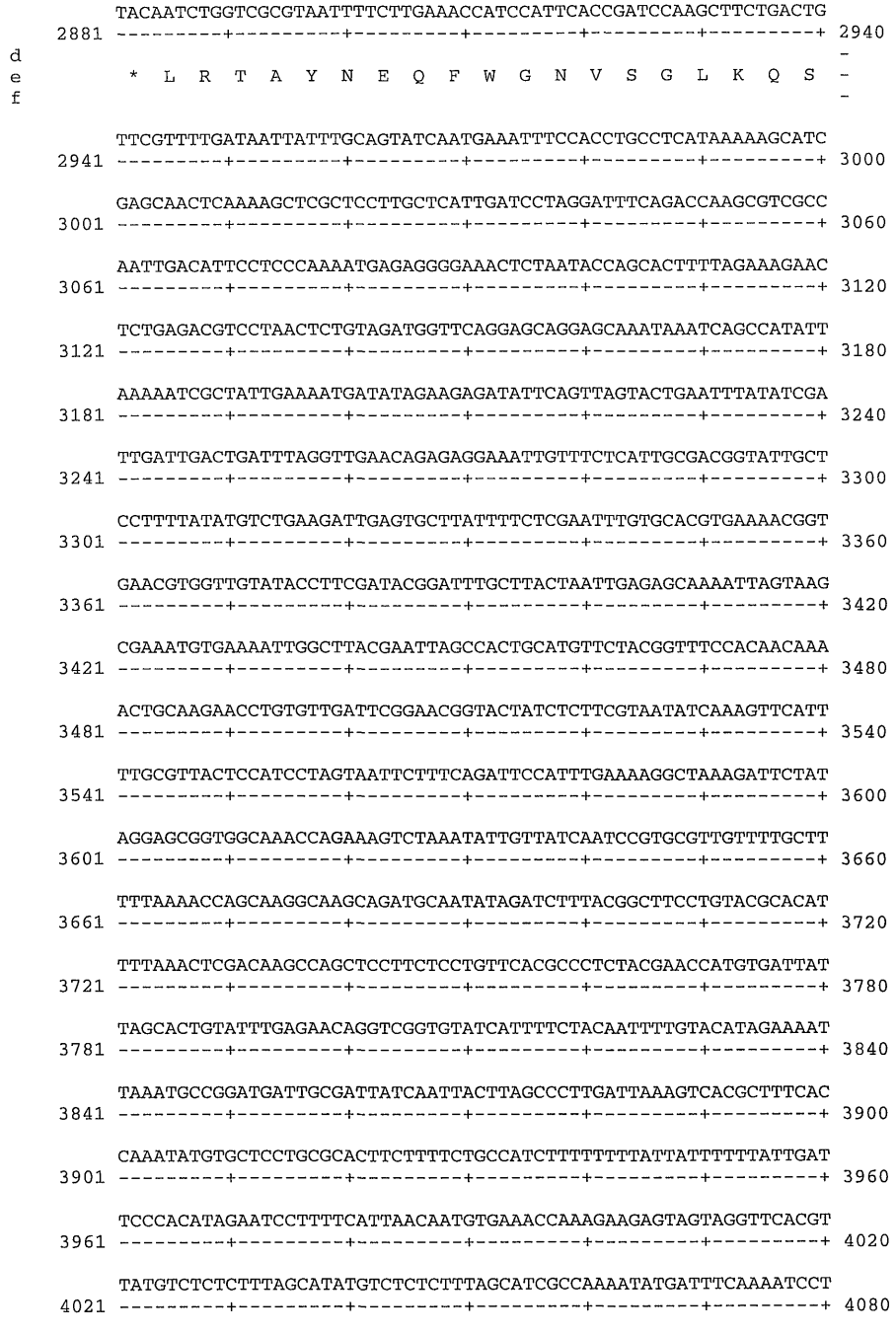
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f
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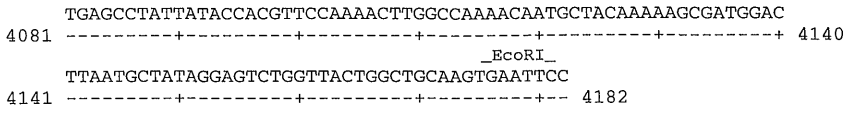
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4141 ---------+---------+---------+---------+-- 4182

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