Regulation of Thiamine Biosynthesis in *Saccharomyces cerevisiae*

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By

ROBERT BURROWS

Department of Genetics
University of Leicester

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ABSTRACT

This study has examined the regulation of thiamine biosynthesis in the bakers' yeast *Saccharomyces cerevisiae*. To facilitate this, promoters have been recovered from *THI5* and *THI12* and fused in-frame with a *lacZ* reporter gene on a low copy plasmid vector. Along with a previously constructed *THI4-lacZ* translational fusion, these constructs enabled *THI4*, *THI5* and *THI12* expression to be monitored by assaying for β-galactosidase activity under defined concentrations of thiamine. It has been shown that *THI4*, *THI5* and *THI12* expression is controlled by the intracellular concentration of the end product of thiamine biosynthesis, thiamine pyrophosphate (TPP). Transcriptional activation of *THI4*, *THI5* and *THI12* has been shown to be dependent upon the activator genes *THI2* and *THI3*; additional work in this laboratory has shown that expression of these genes is also dependent upon *PDC2*. The analysis of *THI12-lacZ* expression also provided the first direct evidence that a second member of the *THI5*-like gene family was transcriptionally active.

A successful screen was developed for the isolation of a new class of mutant strain that displayed derepressed expression of a *THI4-lacZ* reporter gene in medium containing thiamine. Eight single gene *det* mutants were isolated, of which five were due to recessive mutations and three to partially dominant mutations. Phenotypic analysis was carried out on the three partially dominant *det* mutants and one recessive *det* mutant, as these strains displayed derepressed *THI4-lacZ* expression levels comparable to a wild-type *DET*+ strain grown in the absence of thiamine (inducing conditions). This showed that the *det* phenotype was not caused by an inability to synthesise or transport thiamine, and that the mutations affected the expression of all thiamine-regulated genes tested. This latter result supports the theory that thiamine genes are controlled by a common set of regulatory factors. Attempts to clone the corresponding *DET* alleles were unsuccessful, although it was shown that they were not allelic to *THI80* or *PDC2*.

Finally, the gene *RPI1* was identified as a multicy copy activator of thiamine genes. This activation was dependent upon *THI2*, *THI3* and *PDC2*. Analysis of an *rpi1::TnHIS3* disruption strain found that it had no thiamine-associated phenotypes, from which it was concluded that *RPI1* has no role in thiamine gene expression when single copy. However it was shown to be homologous to *PDC2*, due to the presence of asparagine-serine rich regions. Through the production of models of thiamine gene regulation, the method of *RPI1* action has been proposed, and the possible identities of the *det* genes have been discussed.
ABBREVIATIONS

AIR  Aminoimidazole ribotide
apb  Alternative pyrimidine biosynthetic
ARB  Asparagine rich box
BME  β-mercaptoethanol
bp   Base pairs
CAT  Chloramphenicol acetyl transferase
cDNA Complementary DNA
CIP  Calf intestinal phosphatase
dATP Deoxyadenosine 5'-triphosphate
dCTP Deoxycytidine 5'-triphosphate
DEAE-Cellulose Diethylaminoethyl cellulose
dGTP Deoxyguanosine 5'-triphosphate
dITP Deoxyinosine 5'-triphosphate
DMF  N,N-Dimethyl formamide
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
dTTP Deoxythymidine 5'-triphosphate
EDTA Ethylenediamine tetra acetic acid
EMS  Ethyl methanesulphonate
HET  4-methyl-5-β-hydroxyethylthiazole
HMP  2-methyl-4-amino-5-hydroxymethylpyrimidine
IMS  Industrial methylated spirits
IPTG Isopropyl-β-D-thiogalactopyranoside
kb   Kilobases
kDa  Kilodaltons
LB   Luria Bertani
MFS  Major facilitator superfamily
MM  Minimal medium
mRNA Messenger ribonucleic acid
nt  Nucleotides
OD  Optical density
OLB  Oligonucleotide labelling buffer
ONPG o-Nitrophenyl-β-D-galactopyranoside
ORF  Open reading frame
PAGE Polyacrylamide gel electrophoresis
PCR  Polymerase chain reaction
PDC  Pyruvate decarboxylase
PDH  Pyruvate dehydrogenase
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<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PYK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Synthetic dextrose</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>TMP</td>
<td>Thiamine monophosphate</td>
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<td>TPP</td>
<td>Thiamine pyrophosphate</td>
</tr>
<tr>
<td>T-rAPase</td>
<td>Thiamine-repressible acid phosphatase</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-Triphenyltetrazolium chloride</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation site</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>YEPD</td>
<td>Yeast extract peptone D-glucose</td>
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<td>YLE</td>
<td>Yeast lytic enzyme</td>
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CHAPTER ONE
INTRODUCTION

1.1 Thiamine

Thiamine, or vitamin B1, is the oldest known vitamin having been isolated in crystalline form from rice polishings in 1926 by Jansen and Donath (Goodwin, 1963). It is an essential factor in the nutrition of organisms that are unable to synthesise the vitamin de novo, namely animals and some plants and micro-organisms (Haj-Ahm ad et al, 1992). This is because, in the form of its pyrophosphate ester thiamine pyrophosphate (TPP), it acts as a cofactor for a number of enzymes which have functions central to carbohydrate metabolism; these enzymes include pyruvate decarboxylase, pyruvate dehydrogenase, pyruvate oxidase, α-ketoglutarate dehydrogenase, aceto-hydroxyacid synthase and transketolase. In each of these cases, the enzyme bound TPP serves as an intermediate carrier of an activated aldehyde group between metabolites (Leder, 1975). For example when coupled with the pyruvate dehydrogenase complex in the conversion of pyruvate to acetyl CoA, TPP acts in the first stage of the reaction involving the decarboxylation of pyruvate and the transfer of hydroxyethyl onto a second cofactor, lipoamide (Koike and Koike, 1982):

\[
\text{Pyruvate} + \text{TPP} \rightarrow \text{hydroxyethyl-TPP} + \text{CO}_2
\]

\[
\text{Hydroxyethyl-TPP} + \text{lipoamide} \rightarrow \text{TPP} + \text{acetyllipoamide}
\]

These transfers are possible because the carbon atom C-2, present in the thiazole ring of TPP (shown in figure 1.1), is highly acidic and can be deprotonated to form a carbanion that is stabilized by the neighbouring nitrogen and sulphur atoms. TPP is therefore a nucleophile which is able to attack the carbonyl carbon of the different enzyme substrates producing an addition compound with the aldehyde group being transferred. Latest research using NMR techniques indicate that deprotonation of C-2 is dependent upon the protein environment, and in particular on the interaction of a glutamate from the enzyme to which it is bound with N-1 in the pyrimidine ring (Konig et al, 1994; Kern et al, 1997). The above reactions also illustrate the fact that TPP is not degraded, but is instead recycled for further use. This means that TPP is required by the cell in only small quantities.

1.1.1 Human disorders associated with a dietary deficiency of thiamine

Thiamine occurs naturally in a variety of foodstuffs including rice hulls, cereal grains, liver and eggs (Haj-Ahm ad et al, 1992). Suboptimal uptake of the vitamin can lead to a lack of concentration, migraines and depression, however
Figure 1.1 Schematic Drawing of Thiamine Pyrophosphate
Structure of Thiamine pyrophosphate, including the numbering of atoms within the thiazole and pyrimidine rings
severe dietary deficiency can result in the disorders beri-beri and Wernicke-Korsakoff syndrome. Beri-beri is a problem mainly associated with the Far east, where rice forms the staple diet which upon polishing is low in thiamine (Vimokesant et al, 1982). In fact it was during the search for an agent that would cure beri-beri that thiamine was originally discovered in a fraction of rice polishings by Funk in 1911 (Goodwin, 1963). In comparison, Wernicke-Korsakoff is primarily a problem in the western world where it has been associated with alcoholics who are renowned for their poor diets. This disorder is also believed to contain genetic factors, as europeans on thiamine-deficient diets are more susceptible than non-europeans (Stryer, 1988).

An interesting feature of these two disorders is that they result in damage to the nervous system, with beri-beri affecting the peripheral nervous system and Wernicke-Korsakoff affecting the central nervous system (Leder, 1975). At present it is still unclear as to why this should be the case, although two distinct possibilities exist. The first is that due to a lack of thiamine, TPP enzyme activities are reduced and there is a build up of substrate chemicals such as pyruvate and $\alpha$-ketoglutarate. Accumulation of these chemicals could then in turn lead to the damage of nerves. The second possibility and one that is gaining increasing amounts of credence, is that thiamine carries out a secondary function concerned with neural activity (von Muralt, 1962). Some of the early lines of evidence to support this are that in higher organisms, thiamine and its phosphate esters are specifically located in axonal membranes and electrical stimulation and neuroactive drugs provoke the release and hydrolysis of TPP and thiamine triphosphate from within these membranes (Leder, 1975).

Somewhat ironically, a recent study has suggested that too much dietary thiamine can also be potentially damaging to health. Hiramoto et al (1995) found that the combination of thiamine and nitrites at pH3 leads to the production of diazotized compounds, which in turn display mutagenic activity. These conditions would arise in the stomach following the ingestion of foodstuffs such as green vegetables and could be linked with a greater incidence of gastric cancer.

1.1.2 Reasons for studying thiamine in Saccharomyces cerevisiae

There are a number of scientific and commercial reasons for studying thiamine metabolism within the yeast Saccharomyces cerevisiae. Firstly unlike humans, S.cerevisiae is able to synthesis the vitamin de novo. Therefore due to its ease of manipulation and our advanced understanding of its genetics, it has become the model eukaryotic organism for studying biosynthetic pathways, including that of thiamine. This analysis focuses not only upon the steps involved, but also upon the regulation of the pathway at the genetic level.

Knowledge of the thiamine biosynthetic pathway and its regulation is of
economic interest to the food industry for two main reasons. The first is that due to a lack of foodstuffs naturally rich in the vitamin, products such as bread are artificially enriched with thiamine. At present this thiamine is produced by chemical means, which is costly compared to the potential use of yeast strains in the future that have been engineered so as to hyperproduce and excrete thiamine during baking. Similar strains could also be used within the brewing industry to enrich the thiamine content of alcoholic beverages, and in doing so reduce the risk of Wernicke-Korsakoff syndrome. A second use of thiamine within the food industry is that it is a key component of the chemical cocktail that give foodstuffs such as soups their characteristic 'meaty' flavour (Imafidon and Spanier, 1994). Again it would be cost effective to use thiamine enriched extracts produced directly from engineered yeast strains as opposed to chemical supplementation.

Finally as will become apparent, genes involved in the biosynthesis of thiamine are highly expressed and tightly regulated in response to intracellular levels of TPP (Nishimura et al, 1991). This makes the promoters of thiamine genes potentially useful for the production of controllable yeast expression vectors, particularly in studies requiring the use of different carbon sources. This is because at present a large number of controllable yeast expression vectors are based upon the promoters of genes involved in galactose utilisation (GAL1 to GAL10), the fermentative enzyme alcohol dehydrogenase I (ADH1) and the glycolytic enzymes 3-phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GPD) (Schneider and Guarente, 1991). Unlike the thiamine gene promoters, the promoters of these genes are regulated in response to carbon source, making them inappropriate for comparative experiments requiring controlled gene expression in a variety of carbon containing media. Such thiamine controlled expression vectors are already widely used in the fission yeast Schizosaccharomyces pombe, thanks to the production of the pREP and pRIP series of vectors (Maundrell, 1993).

1.2 Thiamine Biosynthesis

Despite being studied intensely over the last 70 years, the complete thiamine biosynthetic pathway has still to be elucidated. The main reason for this relates to the method used for establishing the pathway, the study of incorporation of radiolabelled substrates into thiamine, and the fact that thiamine is present within cells at a very low concentration. Thiamine is present at approximately 30μg per gram of dried cells and does not accumulate in any wild-type organism (Estramareix and David, 1996). This means that it has been extremely difficult to distinguish accurately between the radiolabel that had incorporated specifically into thiamine and the overwhelming levels of unincorporated background label.
An additional and somewhat unexpected problem, was that the pathway appeared to differ between different organisms. For example it was found that radiolabelled formate was incorporated at the C-2 position of the pyrimidine ring of TPP in *E.coli*, and at the C-4 position in *S.cerevisiae* (Kuomoka and Brown, 1967; David *et al*, 1967). This made the solving of the biosynthetic pathway more complicated, as it cast doubt upon the crossover of knowledge gained between different organisms.

1.2.1 The thiamine biosynthetic pathway of *S.cerevisiae*

In 1937, the groups of Schopfer and Robbins showed that the mould *Phycomyces blakesleeanus* required thiamine for growth, but that this requirement could be overcome by the addition of 4-methyl-5-β-hydroxyethylthiazole (HET) and 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) (Schopfer and Jung, 1937; Robbins and Kavanagh, 1937). Following on from this work, numerous fungi were tested for their thiamine requirements and it was found that they needed either (i) thiamine, (ii) HMP, (iii) HET, (iv) HMP and HET, or (v) neither thiamine, HMP or HET; it was into this final class that *S.cerevisiae* fell.

Together these results indicated that thiamine was synthesised from two precursor molecules, namely HMP and HET. This hypothesis was supported by the work of Harris and Yavit (1957) who found that thiamine could be synthesised from HMP and HET in the presence of yeast extracts, ATP and magnesium ions. Subsequently a number of groups have shown that the two precursors require activation by phosphorylation prior to their condensation, with HMP being phosphorylated twice and HET once (Leder, 1959; Nose *et al*, 1959; Lewin and Brown, 1961). The initial product formed by the condensation of HMP and HET is thiamine monophosphate (TMP), which is then dephosphorylated to free thiamine prior to being pyrophosphorylated to produce TPP (Shimazono *et al*, 1959). These steps from the precursors to TPP are shown in figure 1.2, along with the genes known to encode the specific enzyme steps. For those steps at which no gene is listed, the enzyme activities have all been characterized with the exception of the phosphatase at reaction 5. Camienner and Brown (1960) were able to detect this phosphatase activity in crude cell extracts, however upon purification which identified a number of the other thiamine enzymes, the activity was lost. At present it is still unclear as to whether this reaction is carried out by a phosphatase specific to thiamine biosynthesis, or by a more general cellular phosphatase.
Figure 1.2 Thiamine biosynthetic pathway in the yeast *Saccharomyces cerevisiae*. Components of the pathway leading to the formation of thiamine are as indicated. X and Y represent the unknown pathway(s) upstream of the precursors. The enzymes catalysing reactions are as follows: I Hydroxymethylpyrimidine kinase, II Phosphomethylpyrimidine kinase, III Hydroxyethylthiazole kinase/Thiamine phosphate pyrophosphorylase, IV Phosphatase?, V Thiamine pyrophosphate kinase, VI Thiamine-repressible add phosphatase. (adapted from Hather, 1996)
1.2.2 Biosynthesis of the precursors in *S. cerevisiae*

Figure 1.2 includes no information regarding the production of either the HMP or the HET precursor, because little is known about the individual steps leading to their syntheses. In recent years however, radiolabelling studies have provided insight into the probable origins of both of the precursor molecules. In the case of HMP, Tazuya *et al* (1988) have shown that positions N-3, C-4 and the amino N at C-4 are derived from the N-1, C-2 and N-3 atoms of histidine. The remainder of the molecule is produced from pyridoxine, which is itself derived from glutamine and 5-phosphoribosyl-1-amine, as shown in figure 1.3 (Tazuya *et al*, 1993, 1994, 1995a and 1995b). This result is in agreement with earlier incorporation studies which indicated that HMP was derived from ribose, formate and glutamine, and is further evidence for the overlapping biosynthetic pathways of thiamine (vitamin B1) and pyridoxine (vitamin B6). Additional work to suggest that these two pathways were linked was carried out by Kamihara and Nakamura (1982), who found that when thiamine biosynthesis was switched off there was also a decrease in pyridoxine biosynthesis. This implies common biosynthetic enzymes and/or a common regulatory mechanism.

Similar incorporation studies for HET have found that C-2 and the adjacent N originate from glycine and that the remaining five carbon atoms are derived as a unit from a 2-pentulose sugar; this being either ribulose-5-phosphate or xylulose-5-phosphate (White and Spenser, 1979; Grue-Sørensen *et al*, 1985). The origin of the sulphur atom of HET is still uncertain, although it is believed to be gained from cysteine as experiments have ruled out methionine as the source (Estramareix and David, 1996). A proposed scheme for the method of HET formation is also shown in figure 1.3.

1.2.3 Thiamine biosynthetic genes in *S. cerevisiae*

1.2.3.1 THI4 and THI5

As a consequence of our incomplete understanding of the thiamine biosynthetic pathway and in particular those steps leading to precursor formation, it is not surprising that few thiamine biosynthetic genes have been isolated. In this laboratory analysis of thiamine metabolism began somewhat accidentally following a screen looking for genes which were expressed upon entry into stationary phase. Two genes were isolated, *MOL1* and *MOL2* (standing for *MOL*asses inducibility) that displayed transient expression upon entry into stationary phase during growth in molasses medium. This induction was however not observed in complete medium suggesting that this was not growth phase dependent gene expression, but was instead due to the depletion of a nutrient or growth factor from within the medium (Praekelt and Meacock, 1992; Hather, 1996).
Figure 1.3 Schematic drawings of precursor formation in \textit{S.cerevisiae}
Proposed origins of (A) HMP from histidine and pyridoxine (taken from Tazuya \textit{et al}, 1995), (B) HET from glycine, cysteine and a 2-pentulose sugar; either ribulose-5-phosphate or xylulose-5-phosphate (adapted from Estamareix and David, 1996)
Sequence analysis of MOL2 found that it was 62% homologous to the S. pombe gene nmt1(thi3) at the amino acid level (Hather, 1996). The gene nmt1 was known to be involved in the formation of the HMP precursor of thiamine in S. pombe, suggesting a similar role for MOL2 in S. cerevisiae, and that it was a depletion of thiamine within the molasses medium that caused the gene's induction. This indeed appeared to be the case as Northern blot analysis of both MOL2 and MOL1 found that they were expressed in medium lacking thiamine, but were completely repressed throughout batch culture when thiamine was present at concentrations of 1μM or above (Praekelt et al, 1994; Hather, 1996). No other components of the complete medium resulted in MOL1 or MOL2 expression when omitted, therefore the genes were renamed THI4 and THI5 respectively to reflect their potential roles in thiamine metabolism.

Disruption of THI5 did not result in thiamine auxotrophy, as was the case with the S. pombe gene nmt1 (Hather, 1996; Maundrell, 1990). The most likely reason for this difference is functional redundancy, because unlike in S. pombe and also the dairy yeast Kluyveromyces lactis which both contain a single THI5-like gene, S. cerevisiae contains four THI5-like genes. These four genes are THI5 (YFL058w), THI11 (YJR156c), THI12 (YNL332w) and THI13 (YDL244w) and are present in the subtelomeric regions of chromosomes VI, X, XIV and IV respectively (Hather, 1996). This positioning provides a possible explanation for their existence, as they could have been formed by recombinogenic events between the respective chromosomal ends; it has been suggested that the subtelomeric regions play a role in the maintenance of telomeres by recombination when telomerase activity is absent (Louis, 1995). In agreement with such recombination events is the finding that there is a distinct grouping of a set of other genes that are also present at either two, three or all four of these chromosome ends (figure 1.4). What is interesting though is that all four copies of the THI5 gene appear to have been retained intact, with the primary peptides of the four genes being greater than 99% homologous. DNA divergence then begins at approximately 700nt upstream and 100nt downstream from the THI5-like gene coding sequences (Hather, 1996). This lack of divergence compared to the surrounding areas implies that there is some form of selective pressure operating to ensure that all four loci remain functionally active. At present it is unclear as to how many of the four genes are active, although a thi5::LEU2, thi12::TRP1 double disruption strain is still thiamine prototrophic, implying that at least one of the other loci is functional. Work currently underway in this laboratory aims to produce a strain disrupted at all four loci in order to check the phenotype; if the quadruple disruptant is not a thiamine auxotroph, then this would imply an alternative function within the yeast S. cerevisiae.
Figure 1.4 Diagram showing the homologous ORFs within the sub-telomeric regions of chromosomes containing THI5-like genes

Shown are the ORFs and known genes within the sub-telomeric regions of chromosome arms IV-L, VI-L, X-R and XIV-L, that are homologous. This diagram does not show any of the homologies exhibited between the ORFs present at the end of these four chromosome arms, with ORFs present at the ends of other chromosomes.
In comparison with TH15, TH14 is a single copy gene present on chromosome VII which gives rise to a 35kDa protein. A thi4::URA3 disruption strain is a thiamine auxotroph which is rescued by the addition of either thiamine or HET to the medium, indicating a role in the formation of the HET precursor (Praekelt et al, 1994). The deduced amino acid sequence contains a potential adenine dinucleotide binding site suggesting that its function in HET formation is that of an oxidase or dehydrogenase type enzyme (Praekelt and Meacock, 1992). Homology searches using the TH14 amino acid sequence revealed homology to a stress protein of similar size from two species of the plant pathogen Fusarium. Thi4p shows 57.7% identity with Sti35p of F. solani and 59.2% homology with Sti35p from F. oxysporum. (Praekelt and Meacock, 1992). It is unclear as to why this homology exists because strains containing disrupted STI35 genes display increased heat shock and ethanol sensitivity (Choi et al, 1990), which is not the case for thi4::URA3; the sti35 disruption strains have not yet however been tested for thiamine auxotrophy. More recently the identification of TH14 homologues in plants and in particular Arabidopsis thaliana, has also raised the possibility of the Thi4p having an additional stress related role within the cell, in this case that of DNA damage tolerance (Machado et al, 1996). This second possible TH14 function will be discussed in greater detail when describing thiamine biosynthesis in plants (see section 1.2.4.3).

No other genes involved in the biosynthesis of the precursor molecules have been isolated, despite numerous mutagenesis screens for thiamine auxotrophs both in this laboratory and the laboratories of other thiamine groups. These mutagenesis screens have involved the use of various mutagens, including UV light, EMS and transposons, suggesting a limitation in the strategies employed for isolating mutants of precursor synthesis. One possible limitation would be the existence of more than one route for the production of the precursor molecules, as is known to be the case for the production of HMP in Salmonella typhimurium (see section 1.2.4.1). Evidence to support this hypothesis in S. cerevisiae comes from radiolabel incorporation studies carried out by Grue-Sørensen et al (1986), from which they proposed the existence of major and minor routes to HMP synthesis. This work was carried out before Tazuya et al (1995) showed that HMP was derived from histidine and pyridoxine, but what Grue-Sørensen et al (1986) found was that radiolabelled formate could be incorporated at either the C-2 or the C-4 position of HMP. If such alternative precursor biosynthetic pathways do exist, then the isolation of TH14 and TH15 indicates that they must carry out unique roles in the synthesis of HET and HMP respectively.
1.2.3.2 THI6

As shown in figure 1.2, two genes have been isolated that are involved in the biosynthesis of TPP from the precursor molecules. The first is THI6, which encodes a bifunctional enzyme with the HET kinase and thiamine pyrophosphorylase activities (reactions 3 and 4 in figure 1.2). This bifunctionality was known prior to cloning THI6, as it had been found that the two enzyme activities copurified to homogeneity in the same protein (Kawasaki, 1993). To determine whether the different enzyme activities were present within specific domains of the Thi6p, a thi6 null strain was transformed with plasmids containing either sequenced mutant THI6 DNAs or modified THI6 DNAs containing 12-nucleotide linker insertions, and the two enzyme activities assayed (Nosaka et al, 1994). It was found that the 540 amino acid Thi6p could indeed be divided up into functional domains which controlled the different enzyme activities; the regions from amino acids 138 to 187 and amino acids 370 to 453 were essential for the HET kinase and thiamine pyrophosphorylase enzyme activities respectively, whereas the carboxyl terminal region from amino acids 476 to 540 was necessary for both enzyme activities. As it had been shown that the purified Thi6p was an octamer of identical sub units, it was presumed that the carboxyl terminal therefore plays an important role in the oligomerization of Thi6p, which could be a prerequisite for both enzyme activities (Kawasaki, 1993; Nosaka et al, 1994).

The complementation analysis also showed that thi6 null strains carrying a modified THI6 gene displaying thiamine pyrophosphorylase activity but no HET kinase activity, were not thiamine auxotrophs. This implied that HET is not an obligate intermediate in the formation of HET monophosphate, in agreement with earlier work by Kawasaki (1993). It is therefore thought that HET kinase functions in a salvage pathway converting HET into HET monophosphate following the degradation of thiamine within the cell. As this bifunctional enzyme appears to carry out an essential and a non-essential step in the biosynthesis of thiamine, it is unclear as to whether it represents an evolutionary artifice or an artifact. The former would provide increased stability or efficiency for the enzymes, whereas the latter would merely reflect the fusion of gene segments. In E.coli the enzyme activities of ScTHI6 are carried out by two genes, namely thiB and thiM, and these are located within separate thiamine gene clusters (Kawasaki et al, 1968; Mizote and Nakayama, 1989; Mizote et al, 1996).

1.2.3.3 THI80

The final thiamine biosynthetic gene that has been isolated to date is THI80; the method of this isolation will be described in detail in section 1.4.3. The gene THI80 encodes a thiamine pyrophosphokinase and therefore catalyses
the final step in the pathway producing TPP from thiamine (reaction 6 in figure 1.2). Mutations that completely destroy THI80 function are lethal, indicating that the formation of TPP from thiamine is the only way of making TPP within the cell. This confirms the work carried out by Kaziro (1959), and indicates that TPP cannot be made directly from the condensation product thiamine monophosphate.

1.2.3.4 The THI1 mutation

One other mutation that results in a thiamine auxotrophic phenotype has been cited throughout the years, namely thi1 (Hawthorne and Mortimer, 1960). The wild-type allele corresponding to thi1 has been cloned recently within this laboratory and has been shown not to encode an enzyme involved in the biosynthesis of thiamine directly (Byrne, 1997 pers. comm.). The mutation thi1 is instead an allele of ILV2, which encodes the enzyme aceto-hydroxyacid synthase and is involved in the synthesis of the branched side chain amino acids isoleucine and valine (Falco et al., 1985). It does however have a link with thiamine metabolism, in that the Ilv2p is a TPP requiring enzyme. The reason that ILV2 is not believed to have an additional role in thiamine biosynthesis, is that the thiamine auxotrophic phenotype of thi1 could be rescued either by the addition of thiamine, or by the addition of isoleucine and valine to the growth medium. It was therefore hypothesised that thi1 causes a mutation that renders Ilv2p less able to bind TPP, such that in the absence of thiamine the strain is unable to grow as there is insufficient TPP for Ilv2p to function. Reduced aceto-hydroxyacid synthase activity in the thi1 mutant strain has been shown by measuring the production of acetoin, the product of the Ilv2p catalysed reaction, over a range of TPP concentrations compared to similar data from a wild-type strain. At any given TPP concentration less acetoin was produced in the thi1 strain than in the wild-type strain (Byrne, 1997 pers. comm.). It would therefore be more accurate to describe thi1 as an isoleucine-valine auxotroph, as it is not a true thiamine auxotroph.

1.2.4 Thiamine biosynthesis in other organisms

The biosynthesis of thiamine has been studied in a range of organisms other than S.cerevisiae, these being primarily the bacteria E.coli and S.typhimurium, and the fission yeast S.pombe. In recent years however the isolation of thiamine gene homologues has led to increasing amounts of work looking at thiamine metabolism in higher plants. This is particularly interesting because the lack of nutritional mutants in plants has meant that our understanding of the genetic control of their biosynthetic pathways is very poor compared to micro-organisms. Taken collectively this work has shown that
although thiamine is always synthesised from an HMP and an HET precursor, the origin of these precursors and their condensation to form TPP differs between organisms.

1.2.4.1 Thiamine biosynthesis in bacteria

In bacteria radiolabelling studies have shown that the HMP precursor is synthesised not from histidine and pyridoxine, but instead from the compound aminoimidazole ribotide (AIR), a known precursor of purine biosynthesis. These studies also indicated that the AIR used in HMP biosynthesis was produced via the same intermediates as in purine biosynthesis, i.e. ribose, formate and glycine (Goldstein and Brown, 1963; Newell and Tucker, 1968). It therefore appears that the first five steps of de novo purine biosynthesis, which are catalysed by the products of pur genes in the order purF,D,N,G and I, are also common to HMP synthesis. Once the compound AIR has been formed the two pathways diverge to produce their respective end products.

This establishment of AIR as an intermediate in HMP synthesis explained earlier observations from physiological and mutant studies, that purine and thiamine biosynthesis were linked in bacteria. Moyed (1964), and Newell and Tucker (1966), had shown that growth of E.coli and S.typhimurium in the presence of high concentrations of adenine or adenosine inhibited the synthesis of HMP. This is now known to be due to the purR gene product which represses purine biosynthesis and in doing so also represses HMP synthesis. The second observation linking these two pathways was the isolation of E.coli strains containing single site mutations which displayed requirements for both thiamine and purines (Leder, 1975).

The subsequent conversion of AIR to HMP requires the removal of the ribose phosphate, the addition of two single carbon constituents and the opening of the imidazole ring to allow the addition of an extra carbon. Radiolabelling studies indicated that all the carbon and nitrogen atoms within the AIR ring are retained in the same relative positions in HMP, with the exception of AIR C-4 and C-5. These two carbons are derived from glycine which when radiolabelled, was shown to be incorporated at positions C-4 and C-6 in HMP, thus indicating that the extra carbon in the HMP ring is inserted between these two atoms (Newell and Tucker, 1968; Leder, 1975). The current view is that AIR undergoes a novel intramolecular reaction such that the additional carbon atoms are derived from the ribose phosphate portion of the molecule, the remainder of which is removed (Estramareix and Therisod, 1984; Young, 1986).

In addition to this biosynthetic pathway, two other pathways that lead to HMP have also been identified in S.typhimurium, both of which work through the compound AIR. The first is the "purF-independent" pathway, in which as
the name suggests the purF encoded enzymatic step can be bypassed. This pathway is able to operate under aerobic conditions in the presence of pantothenate and requires a functional pentose phosphate pathway to supply ribose-5-phosphate (Downs, 1992; Enos-Belage and Downs, 1996). Recent work also suggests that this pathway is dependent upon the second additional HMP pathway, which is called the "alternative pyrimidine biosynthetic" (apb) pathway (Petersen et al, 1996). This apb pathway bypasses the need for all five pur genes and operates under anaerobic conditions (Downs and Petersen, 1994). An extremely simplified overview of these three HMP biosynthetic pathways is shown in figure 1.5. The presence of these three pathways which utilise metabolites from different sources, increases the metabolic flexibility of the cell enabling thiamine synthesis to occur under a wide range of growth conditions. In order to benefit from this flexibility the cell has to be able to regulate the flow of the shared metabolites such that they are available to the appropriate pathways under a given set of growth conditions (Zilles and Downs, 1996). This regulation could be achieved by branch point enzymes with different affinities for the same substrate, as has been shown for the branch point between the tricarboxylic acid cycle and the glyoxylate shunt (Walsh and Koshland, 1984). The existence of these alternative biosynthetic pathways to a given compound and their tendency to overlap with the biosynthetic pathways of additional compounds, reinforces the potential problems associated with isolating mutations in genes that act at an early stage of a specific pathway.

The HET precursor is also produced differently in bacteria than it is in S.cerevisiae. Radiolabelling studies suggest that the sulphur atom is again derived from cysteine, but that the remainder of the molecule is formed from a different amino acid and 2-pentulose sugar. The C2 and adjacent N are formed from tyrosine instead of glycine and the sugar providing the five carbon unit is 1-deoxy-D-threo-2-pentulose, which is itself produced from pyruvate and a triose phosphate (Estramareix and David, 1996). Curiously, this pentulose has also been shown to be a precursor of pyridoxine in E.coli (Himmeldirk et al, 1996). This means that in bacteria vitamin B1 and B6 are connected via the HET precursor, whereas in S.cerevisiae the vitamins are linked via HMP synthesis. Again a single pathway for the formation of HET is unlikely as it is thought that the derivation of the 2-pentulose sugar varies according to the growth conditions experienced by the cell.

The final known difference between the biosynthesis of thiamine in bacteria and S.cerevisiae, is in the production of TPP from the precursors themselves. Again TMP is formed from HMP pyrophosphate and HET monophosphate but instead of being dephosphorylated to thiamine prior to pyrophosphorylation to TPP, it has been shown that in bacteria TPP is formed
Figure 1.5 Thiamine biosynthesis in *S. typhimurium*

Shown are the three routes to AIR formation during HMP production in *S. typhimurium*. These are (A) the purine biosynthetic pathway with a known branchpoint at AIR, (B) the Pantothenate-dependent or *purF*-independent pathway and (C) the APB pathway.

(Adapted from Peterson et al, 1996)
directly from TMP (Nakayama and Hayashi, 1972a; Nakayama and Hayashi, 1972b). The initial evidence for this difference came from the study of an *E.coli* strain that was already mutant for HMP monophosphate kinase activity. This strain could grow if supplemented with either thiamine, TMP or TPP. On further mutagenesis a mutant strain was isolated that could grow if supplemented with TMP or TPP, but not with free thiamine. Such a mutant strain could not have occurred if the dephosphorylation to thiamine step was an intermediate between TMP and TPP. Nosaka *et al* (1993) have also shown that *E.coli* does not contain the thiamine pyrophosphokinase enzyme activity necessary for the direct thiamine to TPP step. Enzymes have been identified which carry out the thiamine to TMP, and TMP to TPP steps, and are encoded by *thiK* and *thil*, respectively. The thiamine kinase enzyme (*thiK*) is believed to be required for the conversion of thiamine to TMP, prior to its phosphorylation to TPP following the uptake of extracellular thiamine.

1.2.4.2 Thiamine biosynthesis in *S.pombe*

No radiolabelling studies have been carried out in the fission yeast *S.pombe*, therefore it is still unclear as to whether TPP biosynthesis occurs via a similar or completely different pathway to either of those documented for bacteria and *S.cerevisiae*. *S.pombe* gene homologues have been isolated for all the *S.cerevisiae* thiamine biosynthetic genes, including a *THI80* equivalent which encodes the thiamine pyrophosphokinase, indicating that TPP is formed from TMP via a dephosphorylation step to thiamine. The homologous genes are:-

<table>
<thead>
<tr>
<th><em>S.pombe</em></th>
<th><em>S.cerevisiae</em></th>
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<tbody>
<tr>
<td>nmt2 (thi2)</td>
<td>THI4</td>
</tr>
<tr>
<td>nmt1 (thi3)</td>
<td>THI5</td>
</tr>
<tr>
<td>thi4</td>
<td>THI6</td>
</tr>
<tr>
<td>tnr3</td>
<td>THI80</td>
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Despite the existence of *nmt2* and *nmt1* which are homologous to the *S.cerevisiae* precursor biosynthetic genes *THI4* and *THI5*, these homologues do not provide any additional insight into the production of the precursors. This is because plants which also contain homologues of the *S.cerevisiae* gene *THI4*, have been shown to produce HET in a manner more similar to bacteria (see next section). Again what this does imply though, is that *THI4* controls a unique reaction in HET biosynthesis that has been evolutionary conserved between organisms. It will be of interest to see whether the same is true of *THI5* in HMP synthesis.
1.2.4.3 Thiamine biosynthesis in plants

Endogenous thiamine biosynthesis occurs primarily in the leaves of mature plants, and the vitamin is then transported via the phloem to the other parts of the plant that are unable to synthesise it; this includes the roots and shoot apex (Mozafar and Oertli, 1993). Supporting this statement is evidence to suggest that thiamine biosynthesis in plants occurs in the chloroplast. Julliard and Douce (1991) were able to show that chloroplasts contain all the enzymes necessary for the biosynthesis of the HET precursor and for the condensation of HET with HMP to produce thiamine. They also showed that HET was derived from tyrosine, cysteine and 1-deoxy-D-threo-2-pentulose and is therefore most likely synthesised in a manner similar to that in bacteria. As an extension of the work carried out in bacteria, Julliard (1992) was able to provide evidence for the competition of the biosynthetic pathways of HET and pyridoxine for their common intermediate 1-deoxy-D-threo-2-pentulose. This was because pyridoxine is also synthesised in the chloroplast, and by varying the concentration of the precursors of the two vitamins but keeping the concentration of this pentulose constant, he could favour the expression of a given vitamin. For example, pyridoxine requires glycine and HET requires tyrosine; thus by increasing the ratio of glycine to tyrosine, pyridoxine synthesis would be increased and HET synthesis decreased.

At present there is no information regarding the origin of the HMP precursor in plants. However given the apparent similarity between HET synthesis in bacteria and plants, and the view that chloroplasts may have descended from free living prokaryotes (the endosymbiont theory), it will be interesting to see whether HMP is also synthesised via a similar pathway. An additional question raised by this hypothesis, is that if thiamine biosynthesis occurs in the chloroplasts of plants does it therefore occur in the mitochondria of eukaryotes? Some evidence suggests this may be the case as the N-terminus of the \textit{S.cerevisiae THI4} gene is rich in serine, threonine and valine residues, a characteristic of mitochondrial transit signals, or presequences (Hartl \textit{et al}, 1989; Silva-Filho \textit{et al}, 1996). Contrary to this hypothesis though was the finding that of the three plant \textit{THI4} homologues isolated thus far, only the one from \textit{Alnus glutinosa} required its putative chloroplast transit signal to be replaced with a yeast N-terminal leader in order to complement the \textit{S.cerevisiae thi4::URA3} disruption strain (Ribeiro \textit{et al}, 1996). The remaining two \textit{THI4} homologues which were isolated from \textit{Zea mays} (Belanger \textit{et al}, 1995) and \textit{A.thaliana} (Machado \textit{et al}, 1996) and are both called \textit{thi1}, were able to partially complement the \textit{thi4::URA3} yeast mutation without the need for yeast leader sequences. As the \textit{Z.mays} thi1p has been shown to localized within chloroplasts, if \textit{THI4} does function within the mitochondria of yeasts, then this would imply that the thi1
protein was able to be co-targeted to both mitochondria and chloroplasts. This is generally not the case, although it has been shown for the pea enzyme glutathione reductase, which is required for the protection of both mitochondria and chloroplasts from oxidative damage (Creissen et al., 1995).

A second intriguing feature of the A.thaliana thi1 gene, is that it was originally isolated by its ability to complement the defect in DNA base excision repair pathways of the bacterial strain BW535 (Machado et al., 1996). The thi1 cDNA was partially able to complement the methyl methane sulphonate (MMS) sensitivity phenotype of BW535, and partially to correct the UV sensitivity phenotype of the uvrA mutant strain AB1886. This indicated that the A.thaliana thi1p which is 36kDa, and therefore of similar size to the 35kDa Thi4p, carries out dual roles in HET precursor biosynthesis and DNA damage tolerance. This is therefore the second example of THI4 being homologous to a gene required for tolerance to a type of cellular stress, and raises the question as to whether THI4 also possesses an alternative stress-related function(s). It is not yet known whether THI4 is also able to complement the bacterial mutant strains BW535 and AB1886, however it has been shown that THI4 plays a role in mitochondrial DNA damage tolerance. Machado et al (in press) found that a thi4 mutant strain displayed a greater frequency of mitochondrial based respiratory mutants than a wild-type strain following treatment with UV light. This observation supports the view that Thi4p is a bifunctional protein, but also promotes the possibility of Thi4p and therefore thiamine biosynthesis being located within the mitochondria.

1.3 Thiamine uptake in S.cerevisiae

In addition to being able to synthesise thiamine, a number of microorganisms including E.coli and S.cerevisiae have been shown to be capable of acquiring the vitamin from their external environment (Suzuoki, 1955; Kawasaki et al, 1969). This uptake is rapid and highly efficient, enabling large quantities of exogenous thiamine to be accumulated within short periods of time. Praekelt et al (1994) showed that 1µM thiamine was completely sequestered by the S.cerevisiae strain PMY3 within 30 minutes.

Studies involving the measurement of 14C labelled thiamine uptake in S.cerevisiae, indicate that transport occurs via a carrier-mediated active process specific to thiamine and its structural analogues (Iwashima et al, 1973; Iwashima et al, 1975). It was found that thiamine accumulated irreversibly against a large concentration gradient, and that once inside the cell was pyrophosphorylated to TPP (Iwashima et al, 1973; Ruml et al, 1988). This uptake was energy dependent, as transport was stimulated by exogenous glucose and inhibited by metabolic inhibitors such as fluoride and nitride ions (Suzuoki, 1955; Iwashima et al, 1973).
Thiamine transport was also dependent upon pH, temperature and growth phase, with the rate of uptake being maximal during early log phase followed by a gradual decline to approximately one fifth this amount during stationary phase (Iwashima et al, 1979). The thiamine binding activity of growing yeast cells was also at its greatest during early log phase, indicating that the formation and turnover of thiamine-binding proteins correlates with the rate of thiamine transport (Iwashima et al, 1979). The rate of uptake was also related to the external thiamine concentration, with the rate of uptake increasing with the thiamine concentration as expected (Iwashima et al, 1973).

The structural specificity of the thiamine transport system was shown by competition assays in which the ability of cells to transport 14C labelled thiamine was measured in the presence and absence of structurally related compounds. It was found that a 1:1 ratio of the analogues pyrithiamine, chloroethylthiamine and dimethialium with thiamine, reduced thiamine uptake by 42.2, 37.7 and 38.7% respectively at 37°C (Iwashima et al, 1973).

1.3.1 Proteins involved in the uptake of thiamine

Until recently only two thiamine binding proteins had been characterised within *S.cerevisiae*. One of these proteins was known to be present in the soluble fraction of yeast cells, and the other in the membrane fraction. The soluble protein was isolated from yeast cells subjected to cold osmotic shock, by DEAE-cellulose chromatography and ultra filtration, and was found to be a 140kDa glycoprotein present within the periplasm (Iwashima and Nishimura, 1979). The role of this protein in thiamine transport was unknown until Schweingruber et al (1986) showed that production of the acid phosphatase encoded by *PHO3* was repressed by thiamine. Until then *PHO3* was thought to be constitutively expressed, unlike the second known acid phosphatase encoding gene *PHO5*, which is repressible by inorganic phosphate (Toh-e et al, 1973; Toh-e et al, 1975; Bajwa et al, 1984).

Acid phosphatases are cell surface glycoproteins that contain approximately equal amounts of carbohydrates and protein. Their primary role is believed to be the hydrolysis of external phosphate esters that would otherwise be unable to penetrate the plasma membrane (Schweingruber et al, 1986). It was therefore hypothesised that the thiamine-repressible acid phosphatase (T-rAPase) hydrolyses thiamine phosphate esters, the naturally occuring form of thiamine in the environment, prior to uptake of the free vitamin. Also because the secretion of both the soluble thiamine binding protein and T-rAPase was inhibited by tunicamycin, an antibiotic inhibiting glycosylation, that these two proteins were the same (Nosaka et al, 1986). This was first supported by a number of biochemical observations before being proven genetically. The biochemical
evidence to suggest that the 140kDa soluble thiamine binding glycoprotein was identical to T-rAPase was:-
(i) exogenous thiamine resulted in the disappearance of both protein activities.
(ii) the two protein activities co-purified.
(iii) both the native and deglycosylated forms comigrated on PAGE.
(iv) heat treatment gave a similar inactivation profile for both activities (Nosaka et al, 1989a).
The genetic evidence that finally proved that the two proteins were identical, came from the finding that a pho3 mutant defective in T-rAPase activity was also deficient for the soluble thiamine binding activity. These mutant phenotypes also cosegregated upon tetrad analysis of a PHO3/pho3 heterozygous diploid mutant strain (Nosaka et al, 1989b).

Further analysis of a pho3 strain showed that it was able to take up thiamine as well as a wild-type PHO3 strain. However it showed only 24.1% and 14.3% transport activity for thiamine monophosphate and TPP respectively (Nosaka et al, 1989a). Similar results were also gained using yeast protoplasts, indicating that the role of T-rAPase is indeed to hydrolyse thiamine phosphate esters in the periplasm, prior to their transport as free thiamine (Nosaka, 1990). These data also showed that T-rAPase is not directly involved in the transport of thiamine into the cell, which is instead likely to be dependent upon the membrane bound thiamine binding protein. Very little is known about this protein except that it too is glycosylated, and that a strain mutant for it (PT-R2), displays only 3% thiamine binding activity of the parent strain (Iwashima et al, 1979). It was therefore hypothesised that this protein was the actual thiamine transporter, required for the uptake of thiamine as depicted in figure 1.2. Recently however, Singleton (in press) cloned the gene THI7 in a positive selection scheme developed specifically for the isolation of thiamine transporter genes. This gene which is present on chromosome XII, encodes a protein of 598 amino acids that is a member of the major facilitator superfamily (MFS) of transport proteins (Marger and Saier, 1993; Saier, 1994). All MFS proteins are characterized by the presence of twelve membrane-spanning domains, and the family members to which Thi7p is most closely related are the uracil and allantoin permeases (Nelissen et al, 1995; Wergifosse et al, 1994). The need for Thi7 protein in thiamine transport was shown by the fact that a thi7 null strain is unable to grow in the presence of exogenous thiamine, when the thiamine biosynthetic pathway is non-functional. It is not yet known whether the thiamine-binding protein isolated in the membrane fraction of yeast cells by Iwashima et al (1979), is the same protein as that encoded by THI7 (Singleton, in press).
In addition to thi7, Ruml and Silhankova (1996) have also identified a mutation, thp1, that results in a strain which is unable to transport thiamine. This mutation has been mapped to the left arm of chromosome VII, and so is known to be different to THI7, although it might be allelic to the mutation in PT-R2 (Iwashima et al, 1979). It is of course possible that this mutation defines a gene that is not directly involved in thiamine transport, but is instead involved in the regulation of transport. Whichever of these possibilities is correct, the part of figure 1.2 depicting thiamine uptake is oversimplified, in that at least three genes appear to be required for transport of the vitamin.

1.3.2 Uptake of the thiamine precursors HMP and HET

Investigations have also been carried out into the uptake of the two thiamine precursor molecules. HMP uptake is believed to occur via the thiamine transport system, as its transport is inhibited by the addition of the same thiamine-related compounds that block thiamine transport (Iwashima et al, 1990). This result also suggests that it is the pyrimidine ring of thiamine that is recognised by the uptake proteins. One noticeable difference is that unlike thiamine, accumulation of high levels of HMP was followed by leakage of HMP back out of the cells (Iwashima et al, 1990).

In comparison, no specific transport pathway is thought to exist for HET. Instead this molecule is believed to be taken up by diffusion, followed by metabolic trapping within the cell by HET kinase phosphorylation (Iwashima et al, 1986). Such a method for retaining HET could explain the need for the HET kinase activity of the Thi6p, and its bifunctionality. This is because the two enzyme functions of Thi6p would condense any HET taken up by the cell with HMP pyrophosphate to form TMP, which would then be stable within the cell.

1.4 Regulation of thiamine metabolism

As with any metabolic process, the biosynthesis and transport of thiamine requires regulation in order to prevent energy wastage by the cell. This is particularly true in the case of thiamine, because being an enzyme cofactor that is recycled and does not show high levels of degradation, it is only needed in very small quantities. The energy demands of thiamine biosynthesis were shown by Soumalainen and Oura (1971) when comparing the growth rates of yeast strains grown in medium with and without thiamine. They found that in the absence of thiamine the mean doubling time during exponential phase was approximately 3 hours, compared to only 2.5 hours in the presence of thiamine. This reduction in doubling time is due to the cell no longer having to synthesise the vitamin, coupled with thiamine transport requiring less energy than biosynthesis.
1.4.1 Regulation of thiamine transport

In 1976 Iwashima and Nose found that growing cells in the presence of thiamine resulted in a dramatic decrease in their ability to bind and transport further thiamine added to the medium; growth in the presence of 0.1μM and 0.5μM thiamine reduced thiamine uptake by 66 and 93% respectively. Associated with this decrease in transport, was the finding that both T-rAPase and the membrane bound thiamine-binding proteins were absent from cells grown in the presence of thiamine (Iwashima et al., 1979). In the case of T-rAPase, Northern blot analysis of PHO3 mRNA production has shown that this regulation occurs at the level of transcription. PHO3 mRNA was abundant in cells grown in the absence of thiamine and almost completely absent in the presence of thiamine (Nosaka et al., 1993). Therefore production of the thiamine transport proteins was repressed by thiamine, such that transport would be reduced as the transport proteins already present became diluted out as the culture grew. Similar findings have been observed for the transport of other vitamins and amino acids, including biotin and leucine (Rogers and Lichstein, 1969; Cicmanec and Lichstein, 1974; Bussey and Umbarger, 1970).

These studies found that in addition to transcriptional repression, one of two other regulatory mechanisms were employed to gain a more immediate effect on transport. The first is the exit system whereby a transported compound which is beginning to accumulate in excess intracellularly is released back into the medium; an example of this being the vitamin biotin (Cicmanec and Lichstein, 1974). This has however already been shown not to be the case for thiamine, as its transport is irreversible (Ruml et al., 1988). The second mechanism is that of feedback inhibition, whereby the compound being transported inhibits the activity of the transport proteins themselves. This has been shown for the leucine transport system which can be inhibited by the presence of leucine and isoleucine (Bussey and Umbarger, 1970). Initial analysis suggested that thiamine transport is also subject to feedback inhibition. Cells preloaded with thiamine after growth in medium lacking thiamine, displayed reduced rates of thiamine uptake compared to cells which had not been preloaded (Iwashima and Nose, 1976). Additional more conclusive evidence for feedback inhibition of thiamine transport came from thiamine uptake studies carried out in this laboratory. It was found that the addition of high levels of thiamine (40μM) to exponentially growing cells led to a maximal intracellular thiamine concentration of 1600 pmol/10^7 cells within 2 hours, as shown in figure 1.6. The intracellular concentration of thiamine then gradually decreased through dilution as the culture continued to grow. No more thiamine uptake was detected despite the presence of extracellular thiamine and the original
Figure 1.6 Thiamine uptake in *S. cerevisiae*

The *S. cerevisiae* strain PMY3 was pre-grown in the absence of thiamine to a density of 2x10⁶ cells/ml. At time 0, thiamine was added to a final concentration of 40μM and samples were assayed for intracellular and extracellular thiamine over a time course of 10 hours (taken from Praekelt et al, 1994)
thiamine transport machinery, indicating that its activity must have been inhibited (Praekelt et al, 1994).

1.4.2 Regulation of thiamine biosynthesis

In addition to transport, thiamine also regulates its own biosynthesis. Kawasaki et al (1990) found that the activity of the three enzymes involved in forming thiamine monophosphate from HMP and HET (enzymes I to III in figure 1.2) decreased in correlation with increases in the concentration of exogenous thiamine; growth in the presence of 0.5μM thiamine resulted in the complete repression of all four enzyme activities tested. Again Northern blot analysis of \textit{THI6} (encodes enzyme III in figure 1.2) and \textit{THI80} (which encodes the thiamine pyrophosphokinase, enzyme V in figure 1.2), has shown that at least part of this regulation occurs at the level of transcription. Both genes are expressed in the absence of thiamine, and display greatly reduced levels of mRNA in the presence of thiamine (Nosaka et al, 1993; Nosaka et al, 1994). In the case of \textit{THI80}, the level of repression is not as complete as that seen for \textit{THI6}, \textit{PHO3} and the precursor biosynthetic genes \textit{THI4} and \textit{THI5}. Therefore by extrapolating purely from transcript levels, it appears that \textit{THI80} has a higher basal level of expression in the presence of thiamine than the other thiamine biosynthetic genes tested to date. This would be advantageous to the cell as it means that all the intracellular thiamine will be converted into TPP, the active form of the vitamin, even when the rest of the pathway is repressed.

Analysis of \textit{THI4} expression has found that this repression is very rapid; the addition of thiamine to the growth medium at a final concentration of 2μM is such that no \textit{THI4} transcript is detectable within 20 minutes (Praekelt et al, 1994). In \textit{S.cerevisiae} the half lives of expressed mRNAs are known to range from a few minutes to in excess of one hour (Sagliocco et al, 1994). Therefore despite not having measured the half life of \textit{THI4} mRNA, it is clear from the time-scale of this experiment that the \textit{THI4} transcript is very unstable. It has been suggested that the presence of 5' caps and 3'-polyA tails stabilises eukaryotic mRNAs, and that those that are not stable require determinants that act to destabilise them (Brown, 1989). An example of this is the UUUAn motif which is present in the A:U rich 3' untranslated regions of a number of lymphokine, cytokine and proto-oncogene mRNAs (Shaw and Kamen, 1986). Its potential function in mRNA destabilisation was shown by insertion of a sequence containing eight copies of this element into the 3' untranslated region of the rabbit β-globin gene; the half life of the normally very stable β-globin mRNA was reduced from in excess of two hours to approximately 30 minutes. This reduction did not occur when a control insert containing no copies of the UUUAn element was introduced (Shaw and Kamen, 1986). Although these sequences
have not been tested in yeast genes, it is intriguing to note that the 3' untranslated regions of both *THI4* and *THI5* are A:U rich and contain five and four copies of the UUUA element respectively.

A second possibility is that the *THI4* transcript is normally stable, but that it is destabilised in response to the added thiamine. When coupled with the decrease in *THI4* transcription, this increase in *THI4* mRNA turnover would result in the extremely rapid loss of *THI4* mRNA within the cell. A known example of a yeast gene that shows both these levels of regulation is *SUC2*, the structural gene of invertase. Firstly this gene is regulated at the level of transcription in response to carbon source, as it is expressed in the presence of glycerol and repressed in the presence of glucose (Gancedo, 1992; Cereghino and Scheffler, 1996). However it has also been shown that the addition of glucose to a culture previously containing glycerol as the carbon source, greatly increased the rate of *SUC2* mRNA turnover; the half life of *SUC2* mRNA was reduced from 30-45 minutes in the absence of glucose to complete degradation within 20 minutes upon glucose addition (Cereghino and Scheffler, 1996).

To examine the regulation of *THI4* further, a *THI4* promoter-*lacZ* fusion was constructed on the centromeric yeast vector YCp50, as shown in figure 1.7. This plasmid, called pUP39a, contains 1.2kb of *THI4* comprising approximately 1kb of promoter and the first 147bp of the coding region, fused in-frame with the ninth codon of the *E.coli* lacZ gene (Praekelt *et al.*, 1994). As a result expression from the *THI4* promoter could be monitored by assaying for β-galactosidase activity. In agreement with Northern blot data the *THI4-lacZ* reporter gene was constitutively expressed during growth in the absence of thiamine, and repressed in the presence of thiamine; β-galactosidase activities greater than 1000 units were recorded for cells grown in the absence of thiamine compared to less than one unit from cells grown with thiamine (Praekelt *et al.*, 1994). The activity levels of cells grown without thiamine indicate a powerful promoter, as they are comparable to the level of activity recorded from strong promoters such as *GAL1* and *GAL10*. It is uncertain as to why a gene involved in the biosynthesis of thiamine, and possibly DNA damage tolerance, should be expressed at such a high level. A possible explanation for this is that since Thi4p acts at an early stage of thiamine biosynthesis, it has to compete with other biosynthetic pathways for substrate(s). Therefore a high intracellular level of enzymes that function in these early stages would increase the rate of precursor formation, and ensure that TPP is produced as rapidly as possible. This is important because *THI4* is only known to be expressed when levels of this essential vitamin within the cell are low.

The plasmid pUP39a has been used to investigate the kinetics of *THI4* induction as intracellular thiamine levels decrease. Figure 1.8 shows the *THI4*-
Figure 1.7 Plasmid map of pUP39a
Shown is the position and orientation of the THI4-lacZ fusion within the YCp50 vector.
lacZ activity detected in a culture that had been grown in the presence of 2µM, then transferred into thiamine-free medium. Induction of THI4-lacZ occurred at approximately 20pmol/10^7 cells, which is twice the basal intracellular thiamine concentration for this strain grown in the absence of thiamine (Praekelt et al., 1994). This indicates that for THI4, regulation occurs within a very narrow window of total intracellular thiamine concentration. Similar studies in the fission yeast *S.pombe*, have also found that the repression of thiamine metabolic genes occurs within a two-fold increase in intracellular thiamine levels (Fankhauser et al., 1995).

1.4.3 TPP not thiamine is the controller of gene expression

The thiamine pyrophosphokinase gene THI80 (enzyme V in figure 1.2), was cloned by complementation of the thiamine metabolism mutant thi80-1 (Nosaka et al., 1993). This mutant strain was isolated after treatment with the mutagen EMS due to its phenotype of constitutive thiamine transport, as detected using triphenyltetrazolium chloride (TTC) (Nishimura et al., 1991). TTC and other basic dyes such as methylene blue, can be used to monitor thiamine transport as they are taken up using the transport system and reduced within the cell. In the case of TTC, intracellular reduction leads to the production of the red coloured compound formazan. Therefore cells grown in the absence of thiamine readily take up TTC and are red in colour, whereas cells previously grown in the presence of thiamine remain white as the transport pathway is repressed and no TTC is taken up (Iwashima et al., 1981). The thi80-1 mutant gave red colonies in the presence of TTC following growth both with and without thiamine (Nishimura et al., 1991).

Additional phenotypes associated with thi80-1 were the high enzyme activities of T-rAPase and the three enzymes involved in TMP synthesis from the precursors (enzymes I to III in figure 1.2), in both the presence and absence of thiamine. In agreement with this, Northern blot analysis showed that PHO3 and THI6 were transcribed constitutively in the thi80-1 strain. Also the thi80-1 mutant strain showed resistance to the thiamine antagonist oxythiamine, a compound which is normally lethal to cells when in its pyrophosphorylated form. This resistance arose because thiamine pyrophosphokinase activity of the thi80-1 mutant was reduced to only 24% of wild-type. As a consequence of this reduced thiamine pyrophosphokinase activity, intracellular TPP levels following growth in the presence of 0.2µM thiamine were approximately half wild-type, whereas thiamine levels were quadrupled. These data together with the constitutive phenotypes, imply that it is TPP and not free thiamine that acts as the negative effector in the regulation of thiamine metabolism (Nishimura et al., 1991; Nosaka et al., 1993).
**Figure 1.8 Kinetics of THI4-lacZ induction**
Strain PMY3(pUP39a) was grown overnight in the presence of thiamine (2μM), harvested and resuspended in thiamine-free medium. Samples were taken at intervals thereafter and analysed for intracellular thiamine concentration and β-galactosidase activity (taken from Praekelt et al, 1994)
1.4.4 Genes involved in the regulation of thiamine metabolism

To date three genes have been isolated that are involved in the regulation of thiamine genes in response to TPP. These are THI2, THI3 and PDC2 and they are all positively acting regulatory factors, required for the expression of thiamine genes in the absence of thiamine (Kawasaki et al., 1990; Nishimura et al., 1992a; Nishimura et al., 1992b; Richards, 1996). Mutations in any one of these genes results in thiamine auxotrophy.

1.4.4.1 THI2 and THI3

From studies of mutant strains, THI2 and THI3 were known to be required for the activity of T-rAPase, the four isolated biosynthetic enzymes involved in TPP formation (enzymes I to III and V in figure 1.2), and also for the transcriptional expression of PHO3, THI6 and THI80 (Nosaka et al., 1993; Nosaka et al., 1994). The only distinguishing feature between the functions of THI2 and THI3 is in their requirement for thiamine transport. The thi3 mutant strain was reported to be severely reduced in thiamine transport activity, whereas the thi2 mutant strain showed wild-type thiamine transport. This suggested that in a thi2 strain, the protein(s) directly involved in the uptake of thiamine are still being produced (Nishimura et al., 1992b).

By complementation of their thiamine auxotrophic phenotypes, the wild-type alleles corresponding to THI2 and THI3 have been cloned within this laboratory. THI2 is ORF YBR240c, that encodes a protein containing Cys6 zinc finger motifs, a domain characteristic of transcription factors such as GAL4. It is therefore likely that Thi2p positively regulates thiamine genes by directly binding to the gene promoters. In comparison THI3 (YDL080c), encodes a protein that is 52% homologous to the enzyme pyruvate decarboxylase. Links between pyruvate decarboxylase and thiamine metabolism will be discussed in greater detail in the next section (1.4.4.2). However with regard to Thi3p, the homology between these two proteins is fairly extensive and includes those residues that are thought to be required for the binding of TPP. It is therefore a possibility that the role of THI3 in thiamine gene activation, is that of a sensor to detect when TPP levels within the cell are no longer repressing. Whether Thi3p prevents thiamine gene transcription when TPP is bound, or whether free Thi3p is required for thiamine gene activation is as yet unknown.

1.4.4.2 PDC2

The gene PDC2 was recently identified in this laboratory as an activator of thiamine genes, by its ability to complement the thiamine auxotrophic mutants UV2 and UV3. Subsequent Northern blot analysis of a pdc2 mutant strain has shown that it is required for the transcriptional activation of THI4, THI5, THI6,
PDC2 was originally identified by Schmitt and Zimmermann (1982), and cloned and sequenced by Hohmann (1993) as a transcriptional activator of PDC1 and PDC5 expression. These genes are two of the three highly homologous structural genes of pyruvate decarboxylase (PDC), with the third being PDC6 (Hohmann, 1991). PDC is a glycolytic enzyme that catalyses the irreversible cleavage of pyruvate to give acetaldehyde and CO₂, which is subsequently reduced to ethanol by alcohol dehydrogenase. Figure 1.9 shows these reactions and also highlights the fact that pyruvate, the end product of glycolysis, is a branchpoint of two distinct metabolic pathways. As indicated the activities of these pathways are dependent upon environmental conditions, such as the presence of oxygen and the concentration of glucose in the medium. The question therefore arises as to why a gene involved in controlling the production of PDC, should also be involved in controlling thiamine biosynthesis. The most obvious connection is that PDC requires TPP as a cofactor. Therefore when cells require PDC they also require TPP. It could be argued that it might be biochemically expedient to co-ordinate their expression, although if so it would be the first example of enzyme production being controlled by the production of its corresponding cofactor.

In a wild-type strain PDC is produced primarily from PDC1 which is highly expressed, and PDC5 which is weakly expressed. Analysis of PDC1 and PDC5 expression levels using PDC1-lacZ and PDC5-lacZ reporter genes, found that the PDC1 promoter is approximately six times stronger than the PDC5 promoter. In a pdc1Δ strain however, expression from PDC5-lacZ was enhanced four-fold, indicating that a mechanism is operating to ensure that nearly wild-type levels of PDC activity are maintained (Seeboth et al., 1990; Hohmann, 1993). This increase in PDC5 expression is not observed in strains that contain pdc1 point mutations which destroy PDC1 enzyme activity, and in turn accumulate large amounts of the PDC substrate pyruvate (Eberhardt and Hohmann, 1996; Hohmann et al., 1996). This result indicates that the up-regulation of PDC5 is not linked to PDC activity or substrate concentration, but is instead controlled by the PDC protein concentration itself, such that PDCp is able to autoregulate its own synthesis.

Expression of PDC1 is reduced about five fold in a pdc2Δ strain, and PDC5 expression is virtually absent in either a pdc2Δ or a pdc2Δ, pdc1Δ double disruption strain (Hohmann, 1993). Additional work carried out in this laboratory indicates that PDC5 but not PDC1 is also regulated in response to TPP levels, with this expression being dependent upon PDC2. This is because the up-regulation of PDC5 is also observed in a wild-type strain grown in the absence of thiamine, or in a thi80 mutant strain when PDC2 is functional (Richards, pers.
Figure 1.9 Pyruvate degradation under conditions of high and low glucose

Pyruvate is produced from phosphoenolpyruvate by pyruvate kinase (PYK). Under aerobic conditions pyruvate enters the TCA cycle via Acetyl-CoA and under anaerobic conditions it is fermented to produce ethanol via acetaldehyde. This fermentation pathway is however favoured in the presence of high glucose even under aerobic conditions, as the surplus pyruvate which exceeds the capacity of PDH becomes available to PDC (De Deken, 1966; Postma et al, 1989). This aerobic production of ethanol is known as the Crabtree effect (Crabtree, 1929). (Taken from Richards, 1996).
comm). It therefore appears that in the absence of thiamine the cell has developed a mechanism for overproducing PDC, although why this is so is uncertain. Thus far these analyses have only been carried out in the presence of glucose, under which conditions the breakdown of pyruvate occurs primarily through PDC rather than PDH, as shown in figure 1.9. As these two enzyme complexes use TPP as a cofactor, it seems appropriate for PDC to be overexpressed in glucose based medium lacking thiamine, in order for PDC to out compete PDH for the limiting TPP. If however \textit{PDC5} is also up-regulated in medium lacking thiamine and glucose, an alternative explanation for the increase in \textit{PDCp} concentration will be required. At the present time it is unknown whether \textit{PDC5} and \textit{PDC1} expression is dependent on \textit{THI2} and \textit{THI3}.

A role for the third gene \textit{PDC6} has yet to be discovered, although it is uncertain as to whether one exists. This is because \textit{PDC6} is silent in a wild-type strain, and a \textit{pdc1A pdc5A} double mutant shows no detectable PDC activity (Hohmann, 1991; Hohmann and Cederberg, 1990).

Analysis of \textit{PDC2} itself predicts a protein of 925 amino acids, with codon bias index of 0.004 suggesting it is poorly expressed (Bennetzen and Hall, 1982; Sharp and Cowe, 1991; Hohmann, 1993). A striking feature of the deduced amino acid sequence is that it is rich in serine (12% of the residues) and asparagine (11.5% of the residues) (Hohmann, 1993; Raghuram et al, 1994). Homology searches carried out against the SWISSPROT protein database using the BLAST algorithm, revealed homologies with a number of transcription factors. This homology was centred around a region of \textit{PDC2} containing a cluster of asparagine residues (Altshul et al, 1990; Raghuram et al, 1994). Figure 1.10 taken from Raghuram et al (1994), depicts part of this region of homology between \textit{PDC2} and the transcription factors Pho81p and Dal81p from yeast and Mastermind, Caudal and Cut from \textit{Drosophila melanogaster}. The final protein sequence shown in figure 1.10 is that of Rpi1p, which was isolated as a negative regulator of Ras proteins. It was therefore hypothesised that this 'asparagine rich box' (ARB) functions in transcriptional regulation, primarily activation, and that it does so via protein-protein as opposed to protein-DNA interactions. An exact mechanism as to how this might occur is unknown, although one possibility is that it acts in a manner analogous to that of activator proteins containing glutamine-rich regions, where it is proposed that the amide groups form hydrogen bonds with other components of the transcriptional machinery. An example of a glutamine-rich transcriptional activator is \textit{Sp1} in humans (Courey and Tjian, 1988).

More direct evidence to suggest that the ARB functions in transcriptional activation, comes from the analysis of a Gal4-Pdc2p hybrid protein. The N-terminal sequence of Gal4p which contains the DNA binding domain but not the
<table>
<thead>
<tr>
<th>Protein</th>
<th>Length</th>
<th>Sequence Homology</th>
</tr>
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<tbody>
<tr>
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<td>ANEDNNQHLSMASHNP</td>
</tr>
<tr>
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<td>--------------</td>
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<td>-DDDDNNTSSNNK</td>
</tr>
<tr>
<td>RPI1</td>
<td>210</td>
<td>----------------</td>
</tr>
</tbody>
</table>

Figure 1.10 Sequence homology between Pdc2p and other regulatory proteins containing asparagine rich regions

Amino acid residues identical to those in Pdc2p are shown in black boxes. The location of residues within the complete protein sequence is indicated by the number preceding the amino acid sequence.

(Taken from Raghuram et al, 1994)
transcriptional activation domain, was fused in-frame with the C-terminal region of Pdc2p containing the ARB. This hybrid protein, unlike the shortened Gal4p by itself, was able to activate transcription from an integrated GAL1-lacZ fusion gene (Raghuram et al, 1994).

1.4.5 Promoter elements concerned with regulating thiamine gene expression

In an attempt to identify cis elements that act to control expression from the promoters of thiamine genes, promoter analysis has been carried out using deletion studies and homology searches. In this laboratory the THI4 promoter has been studied by producing a series of constructs that contain 5' deletions of increasing size within the THI4 promoter present on plasmid pUP39a (Byrne, pers. comm.). The β-galactosidase activities of these constructs were measured after growth of the strain PMY3 in the presence and absence of thiamine; the results are shown in figure 1.11. From these data it is clear that potential upstream activation sites exist between bases -595 to -365 and -365 to -286 within the THI4 promoter; deletion of these regions led to dramatic decreases in the ability of the THI4 promoter to activate lacZ expression. As all of the constructs tested still showed the ability to repress lacZ expression in the presence of thiamine, this analysis did not provide any evidence for the existence of negative cis-acting regulatory sites.

Similar deletion analysis has been carried out on the PHO3 promoter by Nosaka et al (1992). Their data indicated that the region between bases -234 to -215 is essential not only for the activation of PHO3 in the absence of thiamine, but also for the repression of PHO3 in the presence of thiamine. This was evidenced by the fact that constructs lacking this region displayed comparable levels of expression both in the presence and absence of thiamine, although expression levels were much lower than in the normally induced state. Using electrophoretic mobility shift assays and an oligonucleotide spanning bases -234 to -215, Nosaka et al (1992) went on to show that in the absence of thiamine there was a protein complex specifically bound to this region. It has not yet been determined what these complex is, or whether it differs in strains carrying mutations in any of the known thiamine regulatory genes.

Additional information regarding the location of possible cis-acting elements within thiamine gene promoters, comes from comparing the DNA sequences of the known thiamine genes. Nosaka et al (1994) found that the eight nucleotide sequence AAAATCAA which is repeated in the THI6 promoter at positions -258 to -251 and -131 to -124, is also present in the promoters of PHO3 and THI4. Interestingly in both PHO3 and THI4, the position of this sequence lies within regions of the promoter previously identified by deletion analysis as being involved in regulating gene expression; in PHO3 it is present at -258 to -251 and
Figure 1.11 THI4 promoter analysis
A series of constructs containing increasing 5' deletions of the THI4 promoter and the first 59 amino acids fused in-frame with the *E. coli* lacZ gene were transformed into the wild-type yeast strain PMY3. β-galactosidase assays were then carried out on the PMY3 transformants in medium containing (1.5μM) and lacking thiamine (data source, K. Byrne).
-223 to -216, and in THI4 it is present at -286 to -279. A similar sequence, AAAATCGAA is present at -179 to -171 in THI80 (Nosaka et al, 1994). As Northern blot analysis has shown that THI80 displays a greater basal level of expression in the presence of thiamine, it has been postulated that this incomplete repression is caused by the imperfect sequence motif (Nosaka et al, 1993; Nosaka et al, 1994). However neither of these sequences are present within promoters of the THI5 gene family or PDC5, therefore their role in TPP controlled gene regulation is still very much hypothetical (Hather, 1996).

1.4.6 Regulation of thiamine metabolism in the fission yeast S.pombe

As in S.cerevisiae, the biosynthesis and uptake of thiamine in S.pombe is regulated in response to the end product TPP (Fankhauser et al, 1995). In addition it has been shown that the intracellular concentration of TPP influences sexual agglutination and the ability of cells to form zygotes. Schweingruber and Edenharter (1990) found that growing haploid cells of opposite mating type in minimal medium containing 0.5μM thiamine resulted in a 45% reduction in their subsequent ability to form zygotes; growth in the presence of 1μM thiamine was sufficient to inhibit zygote formation by almost 100%. This inhibition was not as great when the minimal medium base contained even lower concentrations of glucose, phosphate or nitrogen, indicating that TPP levels did not completely override the starvation signals which normally lead to zygote formation. However the effect of thiamine is still striking, especially since it was the only component of complete medium tested, that could significantly affect zygote formation by itself (Schweinguber et al, 1990).

The evidence suggesting that it was TPP and not free thiamine that was the controlling factor in this regulation, again came from the study of a strain mutant for the thiamine pyrophosphokinase gene, which in S.pombe is called tnr3 (Fankhauser et al, 1995). This strain which contained reduced TPP and increased thiamine levels, displayed a greater ability to form zygotes than a corresponding wild-type strain. These data imply that a gene(s) involved in zygote formation is regulated by TPP levels and in agreement with observations from other organisms, is yet another example of a thiamine regulated gene carrying out a function that is related to stress tolerance by the cell.

To date only one gene has been cloned in S.pombe that is involved in the regulation of thiamine genes, namely thi1. This gene is a positive acting regulatory factor required for thiamine biosynthesis and uptake, and was isolated independently by two different groups. Schweingruber et al (1992) isolated thi1 in the more orthodox manner following a screen to identify mutants displaying an inability to express T-rAPase in the absence of thiamine. Such a screen is possible because in the presence of acid phosphatase activity, the compounds α-naphthyl-
phosphate and Fast blue salt are broken down to produce a metabolite which colours the cells red (Toh-e et al., 1973). Therefore in solid medium containing these two compounds and high inorganic phosphate to repress the PHO5 acid phosphatase, it is possible to determine whether the T-rAPase is being expressed by colony colouration. thil was cloned by complementation of a mutant strain which displayed white colonies in the absence of thiamine, as it was found to restore wild-type T-rAPase activity and therefore red colonies (Fankhauser and Schweingruber, 1994). Isolation of thil by the second group was somewhat fortuitous, as it occurred in a screen concerned not with thiamine gene regulation, but instead with identifying factors involved in mitotic initiation (Tang et al., 1994). The strain being studied contained cdc2-4w and wee1-50 mutations, but also an integrated copy of wee1+ under the control of the thiamine regulated promter nmt1. The combination of cdc2-4w and wee1-50 mutations resulted in the premature activation of cdc2/cyclin B kinase, inappropriate initiation of mitosis (a phenotype termed mitotic catastrophe), and a dramatic reduction in cell viability at 35.5°C (Russell and Nurse, 1987). This phenotype was alleviated by wee1+, therefore the presence of the nmt1 controlled wee1+ construct meant that mitotic catastrophe only occurred at 35.5°C when thiamine was present and nmt1-wee1+ repressed. A screen was carried out to look for multicopy suppressors of the mitotic catastrophe phenotype. A yeast strain grown at 25°C was transformed in the presence of thiamine with a genomic DNA library based on the multicopy vector pDW232, and viable transformants selected following a shift to 35.5°C. Of the suppressors isolated one was the gene thil (or ntf1+), because it enabled activation of wee1+ from the nmt1 promoter to occur even under the repressing thiamine conditions. Analysis of thil found that like S.cerevisiae THI2, it too encoded a Cys6 zinc finger containing protein. The THIlp is therefore also likely to be a transcription factor that positively regulates thiamine genes by directly binding to the gene promoters.

At the same time as Schweingruber et al. (1992) identified thil by mutagenesis, a similar screen was carried out looking for mutant strains which displayed T-rAPase activity in both the presence and absence of thiamine. Three classes of mutant were isolated which displayed this constitutive thiamine non-repressible APase phenotype, namely tnr1, tnr2 and tnr3. As already stated tnr3 encodes thiamine pyrophosphokinase and is the equivalent of the S.cerevisiae THI80 gene. The remaining two genes are however as yet unidentified, although it is speculated that they represent genes involved in the repression of thiamine metabolism in response to intracellular TPP. This is because in addition to the constitutive APase activity, they are also constitutive for the expression of all known thiamine genes and thiamine transport.
In an attempt to identify cis-acting regulatory elements, the promoters of *S.pombe* thiamine genes have also been studied by deletion analysis and by homology searches. In the cases of *nmt1*, *nmt2*, and *pho4*, all the sequences necessary for regulating expression in response to TPP lie within the 300 bp immediately upstream of the coding region (Manetti *et al.*, 1994; Silvestre and Jacobs, 1997). Deletion analysis of the *pho4* promoter indicated the presence of an upstream activation site between positions -305 to -256; a construct lacking this region displayed a complete inability to activate gene expression in the absence of thiamine (Silvestre and Jacobs, 1997). Deletion of this region, which is approximately 40 bp upstream of the TATA box, did not alleviate gene repression in the presence of thiamine although it was linked with the control of *pho4* expression under repressing conditions. Silvestre and Jacobs (1997) found that moving the -305 to -256 region further away from the *adh* TATA box affected expression levels both in the presence and absence of thiamine, as shown in figure 1.12. From these data it is tempting to suggest that there is also an upstream repression site within this region of the *pho4* promoter, which relies upon close proximity with the TATA box to carry out its function in repressing gene expression. However the lack of correlation between the size of the insertion between the region -305 to -256 and the TATA box, and in particular whether these insertions result in half turns (multiples of 5 bp) or complete turns (multiples of 10 bp) in the DNA helix and their effect on gene expression, do not support this hypothesis. What can be said though is that this region of the *pho4* promoter appears essential not only for activation but also repression of gene expression. Interestingly deletion analysis of PHO3, the *S.cerevisiae* equivalent of *pho4*, also suggested the presence of overlapping or closely linked activation and repression sites (Nosaka *et al.*, 1992). It would therefore be intriguing to see whether a similar effect occurs in the the PHO3 promoter when its upstream activation site is moved further away from the TATA box.

The region immediately upstream of the TATA box in *nmt1* and *nmt2* has also been examined and in both genes there is an identical 11 bp motif within 30 bp of the TATA box (Manetti *et al.*, 1994). This motif is however not present in the *pho4* promoter. Therefore as was the case with homologous promoter elements in *S.cerevisiae*, the role of this motif in gene expression is still purely hypothetical. With regard to the TATA box itself, Basi *et al* (1993) constructed a series of *nmt1* promoter-cat reporter genes that contained stepwise deletions of the TATA box. What they found was that each new deletion caused a concomitant decrease in *nmt1* promoter strength, both in the presence and absence of thiamine; this decrease being determined by a loss of CAT activity in cell extracts and by a reduction in steady state levels of *cat* mRNA. Utilising the wild-type *nmt1* promoter and also these various TATA box mutants, a series of
Figure 1.12 Effect of moving the pho4 UAS away from the TATA box on gene expression

The construct pTG4734 contains the hirudin gene HV2, placed under the control of the adh TATA box and the pho4 UAS (promoter region -305 to -256). The remaining constructs, pTG6729 to pTG5786, were derived from pTG4734 by insertion of random DNA sequences of the lengths shown in-between the UAS and the TATA box. All constructs were transformed into the strain D18 and hirudin secretion measured following growth in the presence (2μM) and absence of thiamine. (data from Silvestre and Jacobs, 1997).
thiamine-repressible expression vectors have been produced, called the pRIP and pREP vectors (Maundrell, 1993; Basi et al, 1993). Not only do these vectors enable controllable expression of cloned genes, but they also provide a convenient method for studying the in vivo effects of gene product dosage on cell physiology.

1.5 Project Aims

The principal aim of this work is to further our understanding about the regulation of *S.cerevisiae* thiamine metabolic genes in response to TPP, the end product of thiamine biosynthesis. This analysis will focus primarily upon the regulation of *THI4* and *THI5*, but will also examine other known thiamine genes. Expression of the thiamine genes can be monitored by Northern blot hybridisation and also by β-galactosidase assays of *lacZ* expression from thiamine promoter-*lacZ* reporter gene constructs. The main areas of work will therefore be:

(i) To construct *THI-lacZ* translational fusions containing promoters derived from each of the four *THI5*-like genes. This will enable us to determine which members of the *THI5* gene family are transcriptionally active.

(ii) At present it is unknown whether *THI4* or any of the *THI5*-like genes are regulated in response to intracellular concentrations of TPP and not free thiamine, and whether this expression is dependent upon the transcriptional activator proteins Thi2p and Thi3p. This analysis can be carried out by assaying thiamine gene expression in strains mutant for *thi80*, *thi2* or *thi3*, compared to a wild-type strain.

(iii) Using the *THI4-lacZ* reporter gene on the plasmid pUP39a, a mutagenesis screen will be developed to identify strains displaying *THI4-lacZ* expression in both the presence and absence of exogenous thiamine. This derepressed expression will be the result of mutations within genes controlling the repression of *THI4*, which can then be cloned by complementation of the mutant phenotype using plasmid-based genomic libraries.

(iv) Due to the identification of *thi1* as a multicopy activator of thiamine genes in *S.pombe* (Tang et al, 1994), a similar screen will be carried out to look for additional thiamine transcriptional activator genes in *S.cerevisiae*. A wild-type strain containing pUP39a will be transformed with a library based on the multicopy vector YEp13, and colonies displaying derepressed *THI4-lacZ* expression in the presence of thiamine selected. Library plasmids from these clones can then be analysed further to determine which gene is causing the derepressed *THI4-lacZ* expression, and whether this gene has a role in thiamine gene regulation when single copy.
The information gained from (i) to (iv), and in particular from the genes isolated, will be used to develop models which depict the transcriptional activation and repression of thiamine genes in \textit{S.cerevisiae}. 
CHAPTER 2
EXPERIMENTAL PROCEDURES

2.1 Growth media and conditions (Sherman, 1991)

Unless otherwise stated, all media were sterilised by autoclaving at 121°C, 15 psi. for 20 min. Solid media contained 2% (w/v) Difco purified agar. *E.coli* strains were grown at 37°C and *S.cerevisiae* strains at 28-30°C.

2.1.1 Luria broth (LB)

*E.coli* strains were grown in complex LB medium consisting of 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl, pH adjusted to 7.2 with NaOH.

2.1.2 *E.coli* transformant selection

Selection of plasmid-carrying *E.coli* cells was achieved by incorporation of ampicillin (50μg/ml stock) in the LB growth medium. For specific selection of recombinant plasmids in *E.coli* by insertional inactivation of the *lacZ* gene, both inducer IPTG and the chromogenic substrate X-gal were added to the medium at a final concentration of 50μg/ml.

2.1.3 Yeast extract peptone (YEP) media

*S.cerevisiae* strains were grown in complex YEPD medium, consisting of 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose (added after autoclaving from a 40% stock).

2.1.4 Synthetic dextrose (SD) media

Selection of plasmid-carrying *S.cerevisiae* cells was achieved by growth in minimal SD medium. This contained 0.67% (w/v) Difco Yeast Nitrogen Base [containing (NH₄)₂SO₄ but not amino acids], 2% (w/v) glucose, and was autoclaved at 110°C for 10 min. To complement auxotrophic marker genes of the strains used, amino acids and bases were added to the medium as follows: L-lysine HCl (30mg/l), L-tryptophan (40mg/l), L-leucine (40mg/l), L-histidine (20mg/l), adenine sulphate (40mg/l) and uracil (20mg/l).

2.1.5 Wickerhams minimal media

When defined concentrations of thiamine were required, e.g. when studying expression from promoter-*lacZ* reporter constructs in liquid medium, strains were grown in Wickerhams minimal medium (Wickerham, 1951). This consisted of a salts mixture [KH₂PO₄ (1g/l), MgSO₄.7H₂O (0.5g/l), NaCl (0.5g/l), CaCl₂.6H₂O (0.5g/l)], 2% (w/v) glucose as a carbon source, (NH₄)₂SO₄ (2.5g/l) as a nitrogen
source, trace elements \([\text{H}_3\text{BO}_3 (8\mu\text{M}), \text{MnSO}_4.4\text{H}_2\text{O} (2\mu\text{M}), \text{ZnSO}_4.7\text{H}_2\text{O} (1\mu\text{M}), \text{FeCl}_3.6\text{H}_2\text{O} (1\mu\text{M}), \text{Na}_2\text{MoO}_4.2\text{H}_2\text{O} (1\mu\text{M}), \text{K} (1\mu\text{M}), \text{CuSO}_4.5\text{H}_2\text{O} (0.1\mu\text{M})]\), and vitamins \([\text{Nicotinic acid (65}\mu\text{M}), \text{Pantothenic acid (25}\mu\text{M}), \text{Pyridoxine (9}\mu\text{M}), \text{Inositol (110}\mu\text{M}), \text{Biotin (1}\mu\text{M}), \text{P-aminobenzoic acid (4}\mu\text{M}), \text{Riboflavin (2}\mu\text{M})]\). Additions of thiamine were from a 1.5mM stock solution to a final concentration as stated in the results chapters. Complementation of auxotrophic marker genes of the strains used were as stated for SD medium (section 2.1.4).

2.1.6 X-gal supplemented media for studying β-galactosidase expression in \textit{S.cerevisiae}

When expression from promoter-\textit{lacZ} reporter constructs was being studied on solid medium, yeast strains were grown in M63 medium containing the chromogenic substrate X-gal (50\mu g/ml), (Clifton \textit{et al}, 1978). This medium was the same as Wickerhams minimal medium (section 2.1.5), except that it contained a different salts mixture to buffer the medium and prevent β-galactosidase enzyme denaturation. The M63 salts mixture contained \(\text{KH}_2\text{PO}_4 (13.6\text{g/l}), (\text{NH}_4)\text{SO}_4 (2\text{g/l}), \text{FeSO}_4.7\text{H}_2\text{O} (0.5\text{mg/l})\) and \(\text{MgSO}_4 (0.2\text{g/l})\), and was adjusted to pH 6.5 with KOH.

2.1.7 Sporulation media

To induce meiosis, diploid yeast strains that had been grown previously in presporulation medium \([0.8\% (\text{w/v}) \text{ yeast extract, 0.3\% (w/v) bacto-peptone and 10\% (w/v) glucose}]\), were transferred to sporulation medium and incubated at 28°C for 4-10 days until tetrads were detected microscopically. The sporulation medium consisted of 1\% (w/v) potassium acetate, 0.1\% (w/v) yeast extract and 0.05 (w/v) glucose. Nutritional auxotrophic marker genes of the diploid strains were complemented by adding one quarter the amount of amino acids recommended for SD medium (section 2.1.4); reduced amounts of amino acids were used to enable growth initially, whilst maintaining the starvation conditions necessary for sporulation.

2.2 Bacterial and yeast strains

2.2.1 Strains used in this study

All \textit{E.coli} and \textit{S.cerevisiae} strains that were used in this study are listed and characterized in Table 2.1.

2.2.2 \textit{E.coli} strain storage

10ml LB containing 50\mu g/ml ampicillin (if necessary) was inoculated with a single bacterial colony and incubated overnight. 1ml of this culture was aliquoted into a cryogenic tube containing 1ml 50\% (v/v) glycerol, the contents mixed, and
frozen in a dry ice/IMS bath for 5 min. Tubes were then transferred to a -80°C freezer for permanent storage. Strain revival was carried out by inoculating 5ml LB with a loopful of the cell suspension and incubation at 37°C overnight.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/Source</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
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<td>XL1-Blue</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17, SupE44, relA1, lac [F proAB, lacI+ΔM15, Tn10(tetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>DH1</td>
<td>recA1, endA1, gyrA96, thr-1, SupE44, relA1, hsdR17 (rK-, mk+)</td>
<td>Hanahan, 1983</td>
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<td>MH1598</td>
<td>As DH1 + IncW plasmid R388::TnHIS3</td>
<td>Sedgwick and Morgan, 1994</td>
</tr>
<tr>
<td>MH1578</td>
<td>recA1, endA1, gyrA96, thr-1, SupE44, relA1, hsdR17 (rK-+, mk+), rpsL</td>
<td>Sedgwick and Morgan, 1994</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W303a</td>
<td>MATα, ade2-1, can1-100, leu2-3-112, trp1-1, ura3-1, his3-11-15</td>
<td>R. Rothstein, Columbia University</td>
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<td>W303α</td>
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<td>R. Rothstein, Columbia University</td>
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<td>KBY4</td>
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<td>CD1</td>
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<td>det1</td>
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<td>det2-13</td>
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<td>det6</td>
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<td>This study</td>
</tr>
</tbody>
</table>
Table 2.1 Bacterial and yeast strains used in this study

2.2.3 S. cerevisiae strain storage

10ml YEPD was inoculated with a single yeast colony and incubated overnight. 1ml of this culture was aliquoted into a cryogenic tube containing 1 ml preservation mix [K$_2$HPO$_4$ (70mM), KH$_2$PO$_4$ (25mM), Na Citrate (5mM), MgSO$_4$.7H$_2$O (1mM), (NH$_4$)$_2$SO$_4$ (15mM) and Glycerol (40% v/v)]. Tubes were then frozen in a dry ice/IMS bath for 5 min and transferred to a -80°C freezer for permanent storage. Strain revival was carried out by rapidly thawing the tube at 37°C for 2 min, inoculating 10ml YEPD with 1 ml of the cell suspension, and incubation at 28°C overnight.

2.3 Bacterial and yeast plasmids

All E.coli and S. cerevisiae plasmids used in this study are listed and characterized in Table 2.2.

2.4 DNA and RNA isolation

2.4.1 Rapid E.coli plasmid DNA mini-preparation

The method used for rapid screening of E.coli clones harbouring potential recombinant plasmids, was a modified version of the alkaline lysis procedure of
Birnboim and Doly (1979), as described in Sambrook et al (1989). The exception to this method was that solution 1 did not contain lysozyme.

<table>
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<th>Plasmid</th>
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<td>pUC18/19</td>
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<td>Yanisch-Perron et al, 1985</td>
</tr>
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<td>PBS II SK+</td>
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<td>Stratagene</td>
</tr>
<tr>
<td>YCp50</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, tet&lt;sup&gt;R&lt;/sup&gt;, CEN4, ARS1, URA3</td>
<td>Rose et al, 1987</td>
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<td>pUP34</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN4, ARS1, URA3, lacZ (lacking its promoter)</td>
<td>U. Praekelt, University of Leicester</td>
</tr>
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<td>pUP39a</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN4, ARS1, URA3, lacZ (THI&lt;sub&gt;4&lt;/sub&gt;-lacZ fusion)</td>
<td>Praekelt and Meacock, 1992</td>
</tr>
<tr>
<td>pRH11</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, THI5</td>
<td>Hather, 1996</td>
</tr>
<tr>
<td>pRB3</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN4, ARS1, URA3, lacZ (THI&lt;sub&gt;5&lt;/sub&gt;-lacZ fusion)</td>
<td>This study</td>
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<td>pSK+1.1</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, f1(+) ori, THI&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Van Dyck et al, 1995</td>
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<td>pRB4</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN4, ARS1, URA3, lacZ (THI&lt;sub&gt;12&lt;/sub&gt;-lacZ fusion)</td>
<td>This study</td>
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<tr>
<td>pLK2</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN4, ARS1, URA3, lacZ (THI&lt;sub&gt;6&lt;/sub&gt;-lacZ fusion)</td>
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<td>Kew, 1996</td>
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<td>pUP40</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN4, ARS1, URA3, lacZ (CYC1-lacZ fusion)</td>
<td>U. Praekelt, University of Leicester</td>
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<td>pBS-Actin</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, ACT1</td>
<td>R. Hather, University of Leicester</td>
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<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, THI&lt;sub&gt;4&lt;/sub&gt; cDNA</td>
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<td>Baldari and Cesareni, 1985</td>
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<td>pLIB1</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, 2µM-ORI/STB, LEU2 (plus random det1 genomic DNA inserts)</td>
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<td>pAN8</td>
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</tr>
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<td>pRS315</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, lacZ, CEN6, ARS4, LEU2</td>
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<td>pRB7</td>
<td>ori, Amp&lt;sup&gt;+&lt;/sup&gt;, CEN6, ARS4, LEU2, THI&lt;sub&gt;80&lt;/sub&gt;</td>
<td>This study</td>
</tr>
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### Table 2.2. Bacterial and yeast plasmids used in this study

#### 2.4.2 Semi-purified *E.coli* plasmid DNA midi-preparation

Larger amounts of semi-purified plasmid DNA were obtained using the P100 Qiagen kits. This combined the alkaline lysis method (Birnboim and Doly, 1979) with ion exchange chromatography, and was carried out according to the manufacturer's instructions.

#### 2.4.3 Highly purified *E.coli* plasmid DNA maxi-preparation

Plasmid DNA of highly purified quality for yeast genomic DNA library transformations, was prepared by buoyant density ultra-centrifugation in CsCl gradients. The method was adapted from Sambrook *et al* (1989).

**Solutions:**

- **SUT:** 25% (w/v) sucrose, 50mM Tris-HCl, pH8.0
- **TES:** 50mM Tris-HCl, 5mM EDTA, 50mM NaCl, pH8.0
- **LS:** 0.2% (v/v) Triton, 100mM EDTA, 20mM Tris-HCl, pH8.0
- **TE:** 10mM Tris-HCl, 1mM EDTA, pH8.0

Bacterial cells grown overnight in 400ml selective medium were pelleted (6,000 rpm, 10 min, 4°C), resuspended in 20ml SUT, re-pelleted as before and suspended in 10ml TES containing freshly made lysozyme (1mg/ml). Following 10 min incubation on ice, 0.3ml 0.5M EDTA (pH8.0) was added and cells were kept on ice for a further 30 min. Clear cell extracts obtained after centrifugation (35,000 rpm, 35 min, 10°C) were phenol extracted by addition of 8ml phenol and 3ml 1M Tris-HCl (pH8.0) for 5 min, followed by phenol-chloroform extraction in 16ml

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pER1</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, PDC2</td>
<td>Richards, 1996</td>
</tr>
<tr>
<td>YEpl3</td>
<td>ori, Amp⁰, Tet⁰, 2μM-ORI/STB, LEU2</td>
<td>Broach and Hicks, 1980</td>
</tr>
<tr>
<td>YEpl3 Library</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2 (plus random AB320 genomic DNA inserts)</td>
<td>Nasmyth and Tatchell, 1980</td>
</tr>
<tr>
<td>pWTS4</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, RPI1, RHO3, Unknown ORF</td>
<td>This study</td>
</tr>
<tr>
<td>pRB5</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, RHO3, Unknown ORF</td>
<td>This study</td>
</tr>
<tr>
<td>pRB6</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, RPI1</td>
<td>This study</td>
</tr>
<tr>
<td>R388::TnHIS3</td>
<td>F plasmid + TnHIS3</td>
<td>Sedgwick and Morgan, 1994</td>
</tr>
<tr>
<td>pRB6Tn8</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, rpi1::TnHIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRB6Tn10</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, rpi1::TnHIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRB6Tn14</td>
<td>ori, Amp⁰, 2μM-ORI/STB, LEU2, rpi1::TnHIS3</td>
<td>This study</td>
</tr>
</tbody>
</table>
phenol/chloroform (1:1 v/v). To the supernatant (10,000 rpm, 10 min, 4°C) was added 2g ammonium acetate and total DNA precipitated in 0.8 volumes cold isopropanol on ice for 30 min. After centrifugation (10,000 rpm, 10 min, 4°C) the pellet was dried at room temperature, dissolved in 5ml TE containing RNase (1mg/ml) and incubated at 37°C for 30 min. A further 16ml TE, 0.2ml EDTA (pH8.0) and 20g ultrapure CsCl (Sigma) were added, and the solution decanted into sorvall TV850 tubes. Following addition of 0.7ml ethidium bromide (10mg/ml) the tubes were filled up with paraffin oil, precisely balanced, crimp sealed and ultracentrifuged (45,000 rpm, 20 hours, 20°C). Plasmid DNA was harvested under UV light using wide bore syringes and ethidium bromide removed by several washes with an equal volume of water-saturated isobutanol. DNA was then precipitated by the addition of 1 volume of water, 2 volumes absolute ethanol and incubation at room temperature for 1 hour. The plasmid DNA was then pelleted (13,000 rpm, 30 min), washed twice in 70% ethanol, air dried and resuspended in 0.5ml TE.

2.4.4 Rapid preparation of plasmid DNA from S.cerevisiae

Crude preparations of S.cerevisiae plasmid DNA for subsequent transformation into E.coli were obtained by lysis of yeast spheroplasts.

Solutions:
BME: 0.9M sorbitol, 0.05M NaPO4 (pH7.5), 0.1% (v/v) β-mercaptoethanol (added just before use)

4-5ml yeast cells grown overnight in selective medium were pelleted for 1 min in a microfuge at 13,000 rpm and resuspended in 800µl BME. 25µl 10mg/ml yeast lytic enzyme (YLE) was added, the tubes mixed by inversion and incubated at 37°C for 30-45 min until cells were spheroplasting. Tubes were placed at 70°C for 20 min to denature the YLE, 200µl 5M K acetate added, mixed and left on ice for 45 min, followed by pelleting in a microfuge at 13,000 rpm for 30 sec. The supernatant was transferred to a fresh tube and the plasmid DNA precipitated with 550µl isopropanol for 5 min. DNA was then pelleted (13,000 rpm, 5 min) washed in 70% ethanol and resuspended in 20µl TE at 65°C for 5 min. 10µl samples could then be used directly for the transformation of E.coli

2.4.5 Rapid preparation of chromosomal DNA from S.cerevisiae

Total chromosomal DNA was prepared from S.cerevisiae as described by Hoffman and Winston (1987). 10ml yeast cultures were processed, the resulting DNA pellets resuspended in 50µl TE and 10µl aliquots subjected to restriction enzyme and Southern analysis.
2.4.6 Preparation of highly purified chromosomal DNA from \textit{S.cerevisiae}

Total chromosomal DNA of highly purified quality, for use in the construction of plasmid based yeast genomic DNA libraries and as a template for Polymerase chain reactions (PCR), was prepared from spheroplasts using a method based on Cryer \textit{et al} (1975).

\textbf{Solutions:}

A: 1.2M sorbitol, 25mM EDTA, pH8.0
B: 1.2M sorbitol, 0.1M Na citrate, 10mM EDTA, pH8.0
C: 3% sarkosyl, 0.5M Tris-HCl, 0.2M EDTA, pH7.6

Yeast cells grown overnight in 10ml YEPD were diluted to $2 \times 10^6$ cells/ml in 50ml YEPD, re-grown to $2 \times 10^7$ cells/ml and pelleted at 6,000 rpm, 5 min. Cells were resuspended in 5ml A, 175μl 1M DTT added, incubated at 30°C for 30 min with shaking, repelleted (6,000 rpm, 5 min) and resuspended in 5ml B. 100μl 10mg/ml YLE was added, samples mixed and incubated at 30°C for 30-45 min with shaking until cells were spheroplasting. After spheroplasting, cells were washed three times in 5ml 1.2M sorbitol (pelleting each time at 5,000 rpm, 5 min) then resuspended in 2ml C. 100μl 2mg/ml proteinase K (made up in C) was added and cells incubated at 55°C for 1 hour. Volume was then made up to 5ml with TE and the lysate extracted three times with phenol/chloroform and twice with chloroform/iso-amyl alcohol until the interface was clear. Nucleic acids were precipitated in 2 volumes cold ethanol for 15 min on ice, and pelleted at 5,000 rpm, 5 min, 4°C. DNA/RNA was resuspended in 500μl TE, 20μl 10mg/ml RNase added, followed by incubation at 37°C for 1-2 hours. The DNA was then reprecipitated in cold ethanol, washed twice in 70% cold ethanol, air dried and resuspended in 250-500μl TE.

2.4.7 Preparation of total RNA from \textit{S.cerevisiae}

Total yeast RNA was isolated using the rapid phenol/SDS extraction protocol described by Schmitt \textit{et al} (1990).

\textbf{Solutions:}

AE: 50mM Na acetate, 10mM EDTA, pH5.3

20ml yeast cultures grown to $2 \times 10^7$ cells/ml in minimal medium (supplemented with thiamine as stated in the results chapters) were harvested by centrifugation (5,000 rpm, 5 min, 4°C) and resuspended in 1ml AE. To 400μl aliquots were added 80μl 10% SDS, and the solution vortexed hard for 30 sec. An equal volume of AE equilibrated phenol was added and re-vortexed for 30 sec. Samples were incubated at 65°C for 4 min, immediately chilled in dry ice/IMS for 3 min until phenol crystals appeared, and the heating-freezing cycle repeated twice more. Samples were then microfuged at 13,000 rpm for 5 min, and the aqueous layer phenol extracted twice. RNA was ethanol precipitated, washed in 70% ethanol and the pellet resuspended in 50μl water.
2.5 Bacterial and yeast transformation

2.5.1 E.coli transformation

Competent E.coli XL1 Blue cells were prepared using the CaCl2 method described by Sambrook et al (1989). 500μl aliquots of competent cells were flash frozen in dry ice/IMS, and stored at -80°C for up to 6 months.

Plasmid DNA (0.5-1.0μg) or DNA ligation mixes (usually half total amount) were added to 100μl competent cells and incubated on ice for 30 min. Cells were then heat shocked at 37°C for 2 min, the volume made up to 1ml with LB and re-incubated at 37°C for 1 hour. Cells were then pelleted in a microfuge (13,000 rpm, 1 min) resuspended in 100μl LB, from which 10 and 90μl aliquots were plated onto selective medium and incubated at 37°C overnight.

2.5.2 One-step low efficiency transformation of S.cerevisiae

Solutions:
One-step buffer: 40% (w/v) PEG3350, 0.2M Li acetate (pH5.0), 0.1M DTT

Yeast cells were grown overnight in 10ml YEPD. 500μl of culture was pelleted in a microfuge (13,000 rpm, 10 sec), resuspended in 100μl one-step buffer and 1-2μg transforming DNA added. Samples were mixed well, incubated at 45°C for 30 min, then plated directly onto selective medium and incubated at 30°C for 2-3 days.

2.5.3 High efficiency transformation of S.cerevisiae

When S.cerevisiae cells were transformed with plasmid-based yeast genomic libraries, a lithium acetate ultra-high efficiency protocol based on Schiestl and Gietz (1989) was used.

Solutions:
TE: 10mM Tris-HCl (pH7.6), 1mM EDTA (pH8.0)
TEL: 100mM Li acetate in TE (pH7.6)
PEG: 40% PEG3350 in TEL.

Yeast cells were grown overnight in 10ml YEPD, diluted to a concentration of 5x10^6 cells/ml in 20ml fresh YEPD and re-grown to 2x10^7 cells/ml. Cells were harvested at 3,000 rpm for 5 min, resuspended in 1ml TE and pelleted at 7,000 rpm for 2 sec in a microfuge. 1ml of TEL was added, the cells repelleted (7,000 rpm, 2 sec) and resuspended gently in 200μl TEL. To 50μl cell aliquots was added 1μg transforming DNA, 7.5μl 10mg/ml single stranded salmon sperm DNA and 300μl freshly made PEG. Samples were mixed, placed on a 'rock and roller' for 20 min, then heat shocked at 42°C for 20 min. Cells were plated onto selective medium, and incubated at 30°C for 3-5 days.
2.6 Nucleic acid electrophoresis and blotting

2.6.1 DNA electrophoresis

**Solutions:**

- **Loading buffer:** 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol
- **TAE:** 40mM Tris base, 27mM Na acetate, 1mM EDTA (pH 8.2)

DNA molecules were analysed by horizontal agarose gel electrophoresis, the concentration of agarose varying from 0.8-1.5% (w/v) depending on DNA fragment size range being analysed. DNA samples were mixed with one quarter volume loading buffer immediately prior to loading onto the gel, and electrophoresed in TAE running buffer containing 100μg/l ethidium bromide. Electrophoresis was performed at 20-90V depending on the agarose concentration and the time span required for resolution of DNA fragments. DNA in the gel was visualised by UV illumination on a transilluminator, and sized by the simultaneous electrophoresis of standard DNA fragments; these being either HindIII digested phage λ DNA, or the 1Kb ladder from Gibco-BRL.

2.6.2 Southern blotting

DNA was transferred from agarose gels to nylon membranes by the method of Southern (1975). After blotting, DNA was immobilised on the membrane in an Amersham UV crosslinker (RPN 2500), using the preset energy setting of 70,000 μJ cm⁻².

2.6.3 RNA electrophoresis

RNA was analysed by horizontal gel electrophoresis on formaldehyde denaturing agarose gels (Lehrach *et al.*, 1977).

**Solutions:**

- **MOPS (x10):** 200mM MOPS, 83mM Na acetate, 1mM EDTA (pH 7.0).
- **Loading buffer:** 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol, 50% (v/v) glycerol, 1mM EDTA

A 1.5% (w/v) agarose gel made in MOPS, containing 3% (v/v) formaldehyde, was immersed in MOPS running buffer immediately prior to electrophoresis. RNA samples were prepared in sterile microfuge tubes by mixing the following:

- RNA (up to 20 μg) 5μl
- MOPS (x5) 2.5μl
- Deionised formamide 10μl
- Formaldehyde 3.5μl

To denature any double stranded RNA, samples were heated at 65°C for 10 min and immediately cooled on ice. 2.5μl loading buffer, and 1μl 1mg/ml ethidium bromide
was added to each sample followed by electrophoresis at 90-100V for 2-3 hours. RNA was then visualised on a UV transilluminator.

2.6.4 Northern blotting (Thomas, 1980)

Solutions:
SSC (x10): 1.5M NaCl, 0.15M Na citrate (pH7.0)

Following electrophoresis, RNA gels required no treatment prior to blotting. The RNA agarose gel was placed upside down on a piece of 3MM paper on sponges in a tray of 10xSSC. Cut to size nylon membrane (Hybond-N, Amersham) was placed on top of the gel, and covered by 3MM paper (2 sheets) and a stack of paper towels (5cm depth). A 500g weight was placed onto the paper towels, and the RNA transferred onto the membrane overnight by capillary action. RNA was fixed on the membrane in an Amersham UV crosslinker (RPN 2500) using the preset energy setting of 70,000µJ cm⁻².

2.6.5 Preparation of high specific activity probes

DNA probes (for hybridisation with both filter bound DNA and RNA) were radiolabelled using random hexanucleotide primers, based on the method of Feinberg and Vogelstein (1983).

Solutions:
O: 1.25M Tris-HCl, 0.125M MgCl₂ (pH8.0)
A: 1ml O, 18µl β-Mercaptoethanol, 5µl each of 100mM dATP, dTTP, dGTP
B: 2M HEPES-NaOH (pH6.6)
C: Hexanucleotides at 90 A₂₆₀/ml in TE (Pharmacia)
OLB: Mixture of A:B:C in the ratio 10:25:15

20-40ng of the DNA fragment being radiolabelled (in 15µl water), was denatured at 100°C for 5 min then immediately cooled on ice. To this was added 5µl OLB, 1µl (1unit) Klenow fragment (Pharmacia), 1µl 10mg/ml BSA, and 2.5µl α³²P-dCTP, the tube mixed and incubated at room temperature for 4-7 hours. Unincorporated nucleotides were removed by passing the reaction mixture through a Sephadex G50 (pre-swollen with TE) column constructed in a polyallomer wool plugged Pasteur pipette, equilibrated with 100µl aliquots of TE. The crude radiolabelled probe mix was applied to the column and eluted with further 100µl aliquots of TE. Fractions of 100µl were collected from the bottom of the column in Eppendorf tubes, and the passage of probe through column monitored using a Geiger counter. A sharp rise in the radioactivity levels of fractions was usually observed after elution of 500µl, with the following five fractions (containing the highest cpm), being pooled for use in hybridisations. Prior to use in hybrisations, the radiolabelled probe was denatured at 100°C for 5 min.
2.6.6 Pre-hybridisation and hybridisation of nylon membranes

Filters were placed in glass tubes containing 20ml Church-Gilbert buffer (0.5M NaH2PO4, 7% SDS, 1mM EDTA, pH7.4) (Church and Gilbert, 1984), and incubated in a Stuart Scientific hybridisation oven at 65°C with rotation at 6 rev/min, for 4-7 hours. Fractionated radiolabelled probe was then added to the filter (still in Church-Gilbert buffer) and reincubated at 6 rev/min at 65°C overnight. Filters were washed three times in 20ml 3xSSC, 0.1%SDS at 65°C, partially dried by blotting on 3MM paper and wrapped in Saran wrap. Autoradiography was carried out at -80°C using X-ray film in an X-ray cassette for a period ranging from a few hours to one week, depending on the amount of radioactivity detected on the filter after washing.

2.7 DNA manipulation and modification

2.7.1 DNA elution from agarose gels

DNA was eluted from agarose gels using the GeneClean kit (Bio 101 Inc.) according to the manufacturer's instructions. Gel slices were melted in 6M NaI at 65°C, the DNA adsorbed onto silica glass beads, washed and subsequently dissociated from the glass beads in TE at 65°C.

2.7.2 DNA restriction endonuclease digestion

All enzymes used for DNA digestion were supplied by Gibco-BRL, New England Biolabs or Pharmacia with their appropriate reaction buffers, and were stored at -20°C. Digestions were carried out according to the manufacturer's recommendations for each given enzyme, and were stopped by the addition of DNA loading buffer to 25% (v/v), or by phenol extraction.

2.7.3 DNA dephosphorylation

DNA dephosphorylation was carried out using calf intestinal alkaline phosphatase (CIP) (Boehringer-Mannheim) in CIP buffer according to the manufacturer's instructions. Reactions were carried out in a total volume of 50μl, containing 1 unit CIP, and were incubated at 37°C for 30-45 min. Dephosphorylated DNA was recovered by phenol extraction and ethanol precipitation.

2.7.4 DNA ligation

T4 DNA ligase (Pharmacia, 8U/μl) was used for the production of plasmid-based yeast genomic libraries, otherwise T4 DNA ligase (Gibco-BRL, 1U/μl) was used in all other ligations. Generally reactions were carried out in a total volume of 20μl, containing 1μl ligase, 4μl T4 ligation buffer [250mM Tris-HCl (pH7.6), 50mM MgCl2, 5mM ATP, 5mM DTT, 25% (w/v) PEG8000], DNA samples being ligated, and were incubated overnight at 15°C. The amount of DNA used to obtain the
optimal vector: insert ratio, according to Dugaiczyk et al (1975), was calculated based on the relative lengths of the two DNA fragments.

2.7.5 Polymerase chain reaction

All oligonucleotides designed and used within this study are shown in Table 2.3.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Target site</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB1</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>THI80 ORF   (-19 to -2)</td>
</tr>
<tr>
<td>RB2</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>THI80 ORF   (+985 to +966)</td>
</tr>
<tr>
<td>RB3</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>PHO3 ORF   (-34 to -17)</td>
</tr>
<tr>
<td>RB4</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>PHO3 ORF   (+1460 to +1443)</td>
</tr>
<tr>
<td>RB5</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>THI5 promoter (-667 to -650)</td>
</tr>
<tr>
<td>RB6</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>THI5 promoter (-86 to +69)</td>
</tr>
<tr>
<td>RB8</td>
<td>GGGGAATTCAAGCTGAATATCAGCC</td>
<td>THI5 promoter (-25 to -2)</td>
</tr>
<tr>
<td>Ka</td>
<td>CGATCATGGGCGACCACA</td>
<td>Tet ORF (348 to 364)</td>
</tr>
<tr>
<td>δ</td>
<td>AGGGGAACTGAGAGCTCTA</td>
<td>TnHIS3 δ LTR (68 to 86)</td>
</tr>
</tbody>
</table>

Table 2.3 List of synthetic oligonucleotides used throughout this study

PCR was used for DNA sequencing (see section 2.7.6), engineering novel restriction enzyme sites into DNA fragments, and for the amplification of DNA fragments which were not amenable to direct cloning. PCR was performed using either a Techne PHC-3 or a Hybaid Omn-E thermal cycler on 20μl reactions (overlaid with liquid paraffin) containing:
- dNTPs (250μM concentration of each dNTP)
- primer DNA (500μg each primer)
- Template DNA (50-100ng for plasmid, 400-600ng genomic template)
- Deep vent polymerase (1 unit, NEB)
- Deep vent reaction buffer (1x, NEB)

A typical reaction profile consisted of a single 95°C 5 min step, followed by 25 cycles of a denaturation step (95°C, 30 sec), an annealing incubation (50-60°C, 30 sec) and an extension incubation (72°C). The length of the extension incubation time was calculated using a rule of 1 min per kb DNA being amplified (Ehrlich, 1989). The products of PCR reactions were visualised following agarose gel electrophoresis.
2.7.6 DNA sequencing

DNA was sequenced on an ABI model 373A DNA Sequencer, following sample preparation using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit.

**Terminator premix:** 1.58μM A-DyeDeoxy, 94.74μM T-DyeDeoxy, 0.42μM G-DyeDeoxy, 47.37μM C-DyeDeoxy, 78.95μM dITP, 15.79μM dATP, dCTP, dTTP, 16.842mM Tris-HCl (pH9.0), 4.21mM (NH₄)₂SO₄, 42.1mM MgCl₂, 0.42 units/μl AmpliTaq DNA polymerase.

Sequencing reactions were carried out in a total volume of 20μl (overlaid with liquid paraffin) containing 9.5μl terminator premix, 1μg template DNA and 3.2pmol primer DNA. The cycling reaction was carried out in a Hybaid Omn-E thermal cycler, and consisted of 25 cycles of a denaturation step (96°C, 30 sec), an annealing incubation (50°C, 15 sec) and an extension incubation (60°C, 4 min). After PCR the reaction mix was isolated from under the paraffin layer by pipetting, made up to 100μl with water and extracted twice in phenol : H₂O : Chloroform (68:18:14). Extension products were precipitated with 15μl 2M Na acetate (pH4.5), 300μl 100% ethanol, washed in 70% ethanol, then analysed on an ABI model 373A DNA Sequencer.

2.8 det1 genomic DNA library formation (pLIB1)

2.8.1 Determination of Sau3A partial digest conditions

To gain det1 genomic DNA fragments of size 5-10 kb, a series of pilot digestions involving a range of Sau3A concentrations for increasing lengths of time were set up as follows:

1μl det1 genomic DNA (~5μg)
1μl 10 x REact buffer 4 (Gibco-BRL)
1μl Sau3A (either 1U/μl, 0.1U/μl or 0.01U/μl)
7μl water
10μl Total

Enzyme dilution from the original 10U/μl Sau3A stock solution was carried out in water, and for each enzyme dilution a reaction was incubated at 37°C for 5, 10, 15 and 20 min. Reactions were stopped by the addition of an equal volume of phenol-chloroform, followed by DNA extraction and precipitation using ethanol and Na acetate. DNA samples were fractionated on a 0.5% agarose gel by electrophoresis and visualised on a UV transilluminator.

To verify that a Sau3A enzyme concentration of 0.1U/μl for 5 min was appropriate, a second set of trial digestions were carried out. The basic reaction mixture as before was used, with a Sau3A concentration of 0.1U/μl, and the reactions incubated at 37°C for 4, 5, 6, 7, 8 and 9 min. Reactions were stopped by phenol-
chloroform extraction, followed by DNA precipitation and fractionation on a 0.5% agarose gel.

2.8.2 Sau3A digestion of det1 genomic DNA and ligation into pEMBLYe30

The actual Sau3A partial digestion of det1 genomic DNA was a 100 fold scale up of the successful pilot digest, and therefore contained:

- 100μl det1 genomic DNA (~500μg)
- 100μl 10 x REact buffer 4 (Gibco-BRL)
- 1μl Sau3A (10U/μl)
- 799μl water
- 1000μl Total

The digest was incubated at 37°C for 5 min, stopped by phenol-chloroform extraction and the precipitated DNA fractionated by 0.5% agarose gel electrophoresis. DNA greater than 5kb in size was then eluted from the gel and refractionated by 0.5% agarose gel electrophoresis. DNA greater than 5kb was again eluted from the gel, resuspended in 25μl TE, and a 1μl aliquot run a 0.5% agarose gel to determine the size range and concentration of the det1 genomic DNA fragments.

The Sau3A digested det1 genomic DNA was ligated into pEMBLYe30 which had been cut with BamHI and phosphatased with CIP. A 1 : 1 ratio of vector : insert was used with 1μl Pharmacia T4 DNA ligase (8U/μl) and 4μl 5 x T4 ligase buffer, in a total volume of 20μl. After incubation overnight at 15°C, one twentieth of the ligation mix was transformed into E.coli and plated onto LB medium containing ampicillin, X-gal and IPTG (all 50μg/ml). The plasmid DNA from potential recombinant clones (white in colour) was examined by restriction enzyme mapping, to check for insertion of det1 genomic DNA of the correct size range into pEMBLYe30. By extrapolating from the number of recombinant clones gained in this transformation, it was possible to determine the total number of clones within the genomic library, called pLIB1. The remainder of the ligation mix was transformed into E.coli and library plasmid DNA prepared using the CsCl maxi preparation (section 2.4.3)

2.9 Mutagenesis

2.9.1 Mutagenesis of S.cerevisiae using UV-irradiation (After Lee et al, 1988)

Yeast cells to be mutated were grown overnight in 10ml YEPD, the culture pelleted at 5,000 rpm for 5 min and resuspended in 1ml water. Cells were then repelleted at 5,000 rpm for 5 min and resuspended in 20ml 0.9% (w/v) KCl in a petri dish. Mutagenesis was carried out at a dose of 0.5 J m⁻² sec⁻¹ for 120 sec, during which time cell suspensions were being gently but continuously stirred. Cells were then harvested (3,000 rpm, 5 min) resuspended in 10ml YEPD and incubated at 30°C for 4-6 hours. Cell densities were calculated using a haemocytometer, the cultures
diluted and plated onto X-gal supplemented minimal medium (containing 1.5\mu M thiamine) to yield 200-300 colonies per plate. Plates were incubated at 30°C for 3-5 days, then screened for mutant blue colonies.

2.9.2 Transposon mutagenesis of plasmid DNA using TnHIS3

To verify that RPII was the multicopy activator of thiamine gene expression, plasmid pRB6 was subjected to TnHIS3 transposon mutagenesis using the method of Sedgwick and Morgan, 1994. The principle behind this procedure is shown in figure 2.1. The target plasmid pRB6 (\textit{Amp}^r) was transformed into the R388::TnHIS3 containing bacterial strain MH1598 (\textit{Sm}^s), to produce the \textit{Amp}^r, \textit{Sm}^s donor strain. The recipient strain was MH1578 (\textit{Amp}^s, \textit{Sm}^r). 10ml LB cultures of the donor and recipient strains were grown overnight from single colonies in the presence of the appropriate antibiotic. 0.1ml of each culture was then used to inoculate separately 10ml LB medium, and the cultures incubated at 37°C until an OD\textsubscript{600} of 0.2 was reached. 2ml of the donor culture was pelleted (5,000 rpm, 5 min), resuspended gently in 1ml recipient cell culture and the mating mixture plated onto a well dried L agar plate and incubated at 37°C for 1 hour. Plates were then flooded with 10ml sterile 10mM MgSO\textsubscript{4}, the resulting cell suspension centrifuged (5,000 rpm, 5 min) and resuspended in 1ml 10mM MgSO\textsubscript{4}. 10\mu l and 100\mu l aliquots were then plated onto L agar containing methicillin (50\mu g/ml), ampicillin (50\mu g/ml) and streptomycin (100\mu g/ml). Colonies growing on this medium contained the pRB6 plasmid into which had transposed TnHIS3.

2.10 \textit{\beta}-galactosidase assays

2.10.1 Assay for \textit{\beta}-galactosidase activity in liquid culture

\textit{\beta}-galactosidase assays were carried out using the method of Reynolds (1989). The \textit{\beta}-galactosidase activity of the wild-type strain W303a(pUP39a) grown in the presence (1.5\mu M) and absence of thiamine was always measured as a control.

\textbf{Solutions:}
- Z Buffer: 60mM Na\textsubscript{2}HPO\textsubscript{4}, 40mM NaH\textsubscript{2}PO\textsubscript{4}, 10mM KCl, 1mM MgSO\textsubscript{4}, 50mM \textit{\beta}-mercaptoethanol (added just before use), pH7.0
- ONPG: 4mg/ml o-nitrophenol-\textit{\beta}-D-galactoside in 100mM KPO\textsubscript{4}, pH7.0

Yeast strains containing promoter-\textit{lacZ} reporter gene constructs were grown overnight in 10ml Wickerhams minimal medium (supplemented with thiamine as stated in the results chapters), until a cell density of 5x10\textsuperscript{6}-10\textsuperscript{7} cells/ml was reached. At this point OD\textsubscript{600} values of the cultures were recorded, 1ml aliquots pelleted in a microfuge (6,000 rpm, 5 min), the cells washed in water and resuspended in 1ml Z Buffer. 50-1000\mu l of this cell suspension was transferred to a fresh tube, the volume made up to 1ml with Z buffer, followed by addition of 50\mu l chloroform and 10\mu l 0.1% SDS. Samples were vortexed for 15 sec using a multitube vortex adapter and
Figure 2.1 Transposon mutagenesis using TnHIS3

In the diagram above, R388::TnHIS3 is the conjugative plasmid and pRB6 is the target plasmid. (A) Duplicative insertion links R388::TnHIS3 in a cointegrate with pRB6. (B) Conjugation transfers the cointegrate from the donor to the recipient cell. (C) Site-specific recombination between the two copies of the transposon in the cointegrate releases a transposed target plasmid and the original conjugative donor plasmid. After mating, selection with streptomycin eliminates the donor cells, and with ampicillin (and methicillin) all the recipient cells are also killed, except for those which have received a transposed target through conjugation. (Taken from Sedgwick and Morgan, 1994)
incubated at 30°C for 15 min to equilibrate. 200µl ONPG was added to each tube, mixed by inversion, re-incubated at 30°C and timing begun. Reactions were stopped by the addition of 450µl 1M Na2CO3 when the solutions had turned yellow, and the time noted. Samples were centrifuged (13,000 rpm, 2 min) and the OD420 of the supernatant measured compared to a Z Buffer/ONPG blank. β-galactosidase activity values were calculated using the following equation:

\[
\text{UNITS} = \frac{1000 \times \text{OD}_{420}}{t \times v \times \text{OD}_{600}}
\]

where \(t = \) reaction time at 30°C (min)

\(v = \) volume of cells used (ml)

2.10.2 Assay for β-galactosidase activity direct from colonies

To identify false positives gained following the transformation of det1(pUP39a) with the YEpl3 genomic library, a rapid colony-based β-galactosidase assay was developed (shown in Chapter 6, figure 6.2). Colonies to be tested, along with det1(pUP39a) and W303a(pUP39a) as positive and negative controls respectively, were toothpicked onto Wickerhams minimal medium containing thiamine (1.5µM) and incubated at 28°C for 2-3 days. Once grown a small amount of each strain was toothpicked into separate eppendorf tubes, followed by addition of 1000µl Z Buffer, 50µl chloroform and 10µl 0.1% SDS. Tubes were vortexed for 15 sec using a multitube vortex adapter, 200µl ONPG added and incubated at room temperature for 30 min. Reactions were stopped by the addition of 450µl Na2CO3, and the colour of each test sample compared to the two control tubes. Any samples which had remained colourless were assayed for β-galactosidase activity quantitatively as in section 2.9.1. Those samples that had turned yellow were discarded.

2.11 Thiamine assays

Total thiamine concentrations (thiamine, TMP and TPP) were measured using the thiochrome method of Tommasino and Maundrell (1991). This involved the construction of a standard calibration curve derived from solutions of known thiamine concentration. 1 ml of 0, 20, 50, 100 and 200nM thiamine solutions (made up in water) were passed through a 0.2µM filter. To 300µl of each filtered solution was added 1µl 0.6N HCl, 2.4ml 100mM Na acetate and 100µl 42mM K3Fe2(CN)6 (freshly made in 7M NaOH). Samples were mixed, incubated at room temperature for 2 min, 10µl H2O2 added, mixed again and reincubated at room temperature for 2 min. 2.8ml isobutanol was added, the tubes mixed and centrifuged at 1,000 rpm for 1 min. The isobutanol layer was then transferred to a fresh tube, an equal volume of isobutanol added and recentrifuged at 1,000 rpm for 1 min. The isobutanol layer was then assayed against an isobutanol blank in a spectrofluorimeter (Shimadzu RF1501) at an excitation wavelength of 385nm and an emission wavelength of 440nm. A
calibration curve was produced from these values plotted against the concentration (nM) of the filtered 300μl thiamine solutions.

2.11.1 Assay to determine intracellular thiamine concentration

Yeast strains to be assayed were grown in Wickerhams minimal medium with (1.5μM) or without thiamine, until a cell density of approximately 1x10^8 cells/ml; the exact cell density was measured using a haemocytometer. Cultures were pelleted (3,000 rpm, 5 min) and the supernatant transferred to a fresh tube for determination of the extracellular thiamine concentration (see section 2.10.2). The cell pellets were washed twice in 100mM Na acetate, resuspended in 0.5ml 0.6N HCl and incubated at room temperature for 5 min. Samples were then microfuged (13,000 rpm, 30 sec) and the supernatant passed through a 0.2μM filter. 300μl aliquots of the filtered supernatant were then treated as the thiamine standard solutions (section 2.10) and the spectrofluorimetric values gained converted into thiamine concentrations (nM) using the calibration curve, and then into pmol/10^7 cells using the known cell density.

2.11.2 Assay to determine extracellular thiamine concentration

The culture supernatants gained in section 2.10.1 were passed through a 0.2μM filter, and the thiamine content of 300μl aliquots determined by following the protocol used for the thiamine standard solutions (section 2.10). Again the spectrofluorometric values gained were converted into thiamine concentrations (nM) using the calibration curve (section 2.10).

2.12 Thermosensitivity assays

2.12.1 Assay for thermosensitivity phenotype on solid medium

Yeast strains being tested for a thermosensitivity phenotype were initially compared to a wild-type control strain using the plate-based method of Kim and Powers (1991). Strains which had been grown previously on YEPD were replica plated onto fresh YEPD medium, heat shocked at 55°C for 0, 10, 20, 30, 40 and 50 min, then left to recover at 28°C for 2 days. A thermosensitivity phenotype would be detected by a reduced viability or an increased time for recovery following incubation at 55°C, compared to the wild-type control.

2.12.2 Assay for thermosensitivity phenotype in liquid medium

Yeast strains being tested for a thermosensitivity phenotype and a wild-type control strain, were grown overnight in 10ml YEPD medium and 100μl aliquots for each culture added to 900μl YEPD medium that had pre-warmed at 55°C. The cell suspensions were incubated at 55°C for 0, 20, 40, 60 and 80 min, at which time points 10μl samples were plated onto YEPD medium and incubated at 28°C for 2 days.
Again a thermosensitivity phenotype would be detected by the test strain showing a reduced viability or an increased time for recovery following incubation at 55°C, compared to the wild-type control.

2.13 Miscellaneous yeast methods

2.13.1 pUP39a plasmid loss from *S. cerevisiae* strains

*S. cerevisiae* strains containing the plasmid pUP39a were grown overnight in 10ml YEPD from a single colony. Cell densities were measured using a haemocytometer, cultures serially diluted in water, and 100μl aliquots plated onto YEPD medium to gain an expected number of 200 colonies per plate. After incubation at 28°C for 2 days, plates were replica plated onto SD medium supplemented with and without uracil. Colonies that grew only in the presence of uracil had lost the pUP39a plasmid, by growth under non-selective conditions.

2.13.2 Diploid formation, sporulation and tetrax dissection

Yeast strains to be mated were grown overnight in separate 10ml YEPD cultures. 5μl of the first strain was then spotted onto SD medium supplemented with amino acids such that neither haploid strain could grow but the resulting diploid strain could. Once the spot had dried, 5μl of the second strain was spotted in the same location and the plate incubated at 28°C for 2 days. As a control, 5μl samples of the two haploid strains by themselves were spotted onto the SD medium. After the 2 days, a loopful of cells from the patch of growth where mating had occurred, was streaked out on fresh SD medium to gain single colonies.

Sporulation of the newly formed diploid strain occured by growth on pre-sporulation medium, followed by starvation on sporulation medium (see section 2.1.7). When tetrads could be detected microscopically, a loopfull of cells was resuspended in 100μl water, 5μl β-glucoronidase (90,000 units/ml, Sigma) added and incubated at room temperature for 5 min to digest the asci walls without dissociating the sets of four spores. 10μl of the suspension was then streaked along one edge of a YEPD plate (poured on a levelling platform) and the meiotic tetrads dissected using a Singer MSM micro-dissection system. YEPD plates containing dissected tetrads were incubated at 28°C for 2 days, and the resulting haploid spores streaked onto fresh YEPD medium prior to analysis of nutritional marker and *det* segregation by plating onto selective SD and X-gal medium respectively.
CHAPTER THREE
FACTORS INVOLVED IN THE REGULATION OF THI4, THI5, AND THI12

3.1 Introduction

At the beginning of this study, two genes that function in the biosynthesis of thiamine had been isolated within this laboratory, these being THI4 and THI5. THI4 is a single copy gene present on chromosome VII, that is thought to encode an oxidase/dehydrogenase enzyme involved in the production of the thiazole precursor HET (Praekelt and Meacock, 1992; Praekelt et al, 1994). In comparison, THI5 is thought to be involved in the formation of the pyrimidine precursor HMP and is a member of a multigene family (Hather, 1996). This gene family consists of four genes, THI5, THI11, THI12 and THI13 that are present on chromosomes VI, X, XIV and IV respectively. The primary polypeptide products all show greater than 99% amino acid sequence identity. Through Northern blot analysis it has been shown that THI4 and THI5 are regulated at the level of transcription; both genes were highly expressed when grown in the absence of thiamine, and completely repressed when grown in the presence of thiamine at a concentration of around 1μM and above (Praekelt et al, 1994; Hather, 1996). No information was known about the activity or regulation of THIU, THI12 or THI13.

Studies by Nishimura et al (1991) found that genes involved in thiamine metabolism were regulated not by free thiamine, but in response to intracellular levels of TPP, the end product of thiamine biosynthesis. Furthermore expression of the thiamine genes was dependent upon two transcriptional activator proteins encoded by THI2 and THI3 (Kawasaki et al, 1990; Nishimura et al, 1992b). This analysis has yet to be tested for THI4 or any members of the THI5 gene family. However available to me were strains carrying either thi2, thi3 or thi80 mutations. Therefore by testing THI4 and THI5 expression in these strains it should be possible to determine whether expression of these genes is also regulated by TPP levels and dependent upon functions encoded by THI2 and THI3. It was decided to monitor expression of THI4 and THI5 by Northern blot analysis, and also by use of promoter-lacZ reporter gene fusions. In the case of THI5 this fusion needed to be constructed. At the same time a reporter gene specific for the THI12 promoter was made, so that expression of two members of the THI5 family could be compared.

In addition I decided to examine THI4, THI5 and THI12 expression during growth in the presence of the thiamine precursors HET and HMP, either individually or together. This experiment would indicate any feedback regulation between the intracellular concentration of a precursor and expression of a gene involved in its production.
3.2 Construction of promoter-reporter gene fusions

Already available was a *THI4-lacZ* reporter gene present on the plasmid pUP39a, as shown in figure 1.7. This plasmid enabled expression of the *THI4* promoter to be measured quantitatively by assaying for β-galactosidase activity in response to growth under defined conditions of thiamine. No such reporter genes existed for any members of the *THI5* gene family, therefore it was necessary to produce a similar construct for *THI5*, and if possible *THI11, THI12*, and *THI13*. Production of these new constructs again involved the fusion of the thiamine gene promoter to *lacZ*, such that gene expression could be measured quantitatively by assaying for β-galactosidase activity.

3.2.1 Construction of a *THI5-lacZ* reporter gene

The *THI4-lacZ* reporter gene was produced by direct cloning of a 1.2Kb *THI4* gene fragment, consisting of the gene promoter and 147bp of coding region, upstream of the *lacZ* gene present on pUP34. A similar procedure was not possible for *THI5*, as this gene did not possess suitable restriction enzyme sites to facilitate cloning into pUP34. A PCR strategy was therefore devised. Primers were designed that were homologous to *THI5* and generated a PCR product 754bp in length, consisting of 667bp of promoter DNA and the first 87bp (29aa) of coding sequence. The primers had the added feature of either an EcoRI or a BflmHI restriction site attached 5' to their respective *THI5* target DNA sequences, as shown in Table 3.1. Thus the resulting PCR product was flanked at the upstream end by an EcoRI site and at the downstream end by a BflmHI site. The PCR product was digested with EcoRI and BflmHI and cloned into similarly cut pUP34 to create an in-frame translational fusion of the *THI5* ORF and *lacZ* on the plasmid pRB3 (figure 3.2). The DNA template used in the PCR reaction was pRH11, a pUC18 based plasmid containing a 4Kb genomic insert spanning the *THI5* gene. A specific plasmid template was required because PCR from genomic DNA would have resulted in a heterogeneous set of PCR products, primed from any of the four genes in the *THI5* family, due to their very high degree of homology. After ligation, the DNA sequence across the *THI5-lacZ* junction was checked to ensure that the correct reading frame from the thiamine gene into *lacZ* had been maintained. The DNA sequence at the point of fusion is shown in figure 3.1.

3.2.2 Construction of a *THI12-lacZ* reporter gene

As stated the homology within the *THI5* gene family meant that construction of *THI11-, THI12- and THI13-lacZ* reporter genes required plasmids containing the individual genes for use as PCR templates. Although plasmids containing either *THI11* or *THI13* were not available, the plasmid pSK+1.1 which
### Table 3.1 Primers used to PCR amplify the THI5 promoter during THI5-lacZ production

Bases that are underlined indicate those that were not homologous to THI5 sequence. Bases in bold highlight the EcoRI site in RB5, and the BamHI site in RB6. The two underlined bases 3' to the BamHI site in RB6, were added to maintain the correct reading frame through the THI5-lacZ junction.

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence (5' --&gt; 3')</th>
<th>Target site within THI5</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB5</td>
<td><strong>GGG GAA TTC</strong> GTA GAA CTA GCG ATG CTC</td>
<td>-667 to -650</td>
</tr>
<tr>
<td>RB6</td>
<td><strong>GGG GAT CCC CGT</strong> AAC CTT TGG TTT GAG C</td>
<td>+70 to +87</td>
</tr>
</tbody>
</table>

### Figure 3.1 DNA sequence across the THI5-lacZ reporter gene junction

The numbers shown above codons indicate their original position in either THI5 or lacZ. Those codons that are not numbered but underlined represent new codons formed by the fusion event itself, with the BamHI site at the point of fusion shown in bold.

```
26 27 28 29  9 10 11 12
5' ACC AAA GGT TAC GGG GAT CCC GTC GTT TTA CAA 3'
3' TGG TTT CCA ATG CCC CTA GGG CAG CAA AAT GTT 5'

Thr Lys Gly Tyr Gly Asp Pro Val Val Leu Gln

THI5-----------------> BamHI lacZ----------------->
```
Figure 3.2 Diagram showing the construction of a THI5-lacZ translational fusion
contained a genomic clone spanning *THI12* was, supplied by A. Goffeau. Therefore by using the same primers and strategy as employed for the *THI5-lacZ* construct production, but with pSK+1.1 as the PCR template, a *THI12-lacZ* reporter gene construct was generated. The plasmid carrying this reporter gene was named pRB4.

3.3 Are *THI4*, *THI5* and *THI12* regulated by TPP levels?

To determine whether *THI4*, *THI5* and *THI12* were regulated by TPP, their expression was compared in the *thi80-1* mutant strain T48-2D and the *THI80* strain YPH500 after growth in the presence and absence of thiamine. Derepressed expression in strain T48-2D but not YPH500 would indicate that the *THI* gene was controlled by intracellular TPP concentrations, and not free thiamine.

3.3.1 Use of *lacZ* reporter constructs to monitor the effect of the *thi80* mutation on *THI* gene expression

The strains T48-2D and YPH500 were transformed with the *lacZ* fusion construct plasmids pUP39a, pRB3 and pRB4, and transformants selected on minimal medium lacking uracil. β-galactosidase assays were then carried out on liquid cultures of the plasmid-containing strains, grown either with thiamine (1.5 μM) or without thiamine (Table 3.2 and in figure 3.3).

As expected from *THI5* Northern blot analysis (Hather, 1996), cultures of YPH500(pRB3) gave high levels of β-galactosidase activity in the absence of thiamine, around 500 units, compared to less than 1 unit in the presence of thiamine. This indicated that a functional reporter gene fusion had been produced containing all the necessary *THI5* promoter sequence for regulation in response to exogenous thiamine. Cultures of YPH500(pRB4) gave very similar expression levels to YPH500(pRB3) both in the presence and absence of thiamine, indicating that a functional *THI12-lacZ* reporter gene had also been produced, and that *THI12* was regulated and expressed in a manner similar to *THI5*. The induced β-galactosidase activity levels observed for *THI5-lacZ* and *THI12-lacZ* were not as great as those recorded from *THI4-lacZ*; YPH500(pUP39a) gave over 800 units of activity when grown in the absence of thiamine. The increased activity of the *THI4-lacZ* construct must reflect either a stronger promoter, or the different N-terminal sequences and structures of the fusion proteins.

All three constructs showed derepressed expression in the *thi80-1* mutant T48-2D grown in the presence of thiamine. The highest expression level was recorded from T48-2D(pUP39a) which gave 75 units of β-galactosidase activity, compared to the 0.5 units gained for the YPH500(pUP39a) control. Strains T48-2D(pRB3) and T48-2D(pRB4) showed slight derepression, both giving
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MM - Thiamine</td>
<td>MM + Thi (1.5μM)</td>
<td></td>
</tr>
<tr>
<td>YPH500(pUP39a)</td>
<td>808 ± 104</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>T48-2D(pUP39a)</td>
<td>707 ± 86</td>
<td>74 ± 17</td>
<td></td>
</tr>
<tr>
<td>YPH500(pRB3)</td>
<td>451 ± 52</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>T48-2D(pRB3)</td>
<td>547 ± 90</td>
<td>5.6 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>YPH500(pRB4)</td>
<td>459 ± 20</td>
<td>0.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>T48-2D(pRB4)</td>
<td>404 ± 28</td>
<td>5.2 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 THI4-, THI5- and THI12-lacZ expression in a thi80 mutant strain

β-galactosidase activity of YPH500 (THI80) and T48-2D (thi80) transformed with pUP39a, pRB3 and pRB4 after growth in medium with (1.5μM) and without thiamine. All assays were carried out in triplicate on duplicate samples.

Figure 3.3 THI4-, THI5- and THI12-lacZ expression in a thi80 mutant strain

Graphical representation of the β-galactosidase activity data from Table 3.2.
approximately 5 units of β-galactosidase activity. Thus THI4, THI5 and THI12 were all regulated in response to intracellular TPP.

### 3.3.2 Analysis of THI gene expression in the thi80 mutant by Northern blot hybridisation

To check that the chromosomal THI4 and THI5 loci were also derepressed in the thi80-1 strain, Northern blot analysis was carried out on RNA isolated from T48-2D and YPH500 grown with (1.5µM) and without thiamine. As an additional control, RNA was isolated from T48-2D(pAN8) grown under the same conditions; the plasmid pAN8 contained the wild-type THI80 gene. Identical filters carrying cellular RNAs were hybridised separately with (i) DNA of the ACT1 gene as a loading control, (ii) the THI4 cDNA and (iii) the THI5 cDNA. Again the homology within the THI5 gene family meant that the THI5 cDNA probe would hybridise with mRNA transcribed from any of the four gene members, even under conditions of high stringency. The results of these Northern blots are shown in figure 3.4.

In agreement with the reporter gene assays (Table 3.2), THI4 was expressed in cultures of T48-2D grown both in the presence and absence of thiamine, but only in cultures of YPH500 grown without thiamine. Wild-type regulation of THI4 expression was restored in T48-2D(pAN8), indicating that THI4 derepression in T48-2D was indeed due to the thi80-1 mutation and that THI4 was regulated in response to intracellular TPP. In the case of THI5, expression was initially detected only in the absence of thiamine in all three strains tested, implying that it was not regulated by TPP. However upon overexposure of the THI5 probed filter, a weak signal did become apparent in samples of RNA from strain T48-2D grown in medium containing thiamine (lane 3). This was not seen in either of the corresponding control strains (lanes 1 and 5). This result was consistent with the weak level of THI5 derepression observed in the T48-2D(pRB3) reporter gene assays (Table 3.2), and indicated that THI5 was also regulated by TPP levels.

### 3.4 Are THI4, THI5 and THI12 regulated in response to precursor concentrations

As THI4, THI5 and THI12 are thought to act in the formation of the thiamine precursors, it was decided to test whether they were also regulated in response to intracellular concentrations of these precursor molecules. The THI+ strain W303a was transformed with plasmids pUP39a, pRB3 and pRB4 and transformants selected on minimal medium lacking uracil. β-galactosidase assays were then carried out on the plasmid-containing strains to measure promoter activity after growth in medium containing (i) no additions, (ii) thiamine (1.5µM), (iii) HMP (2µM), (iv) HET (2µM) and (v) HMP and HET together (both 2µM). The results of these assays are shown in Table 3.3 and figure 3.5.
**Figure 3.4 Northern blot analysis of THI4 and THI5 expression in a thi80 mutant strain**

Total cellular RNAs from strains YPH500 (THI80), T48-2D (thi80) and T48-2D(pAN8) grown with (1.5μM) and without thiamine, were hybridised with probes specific for ACT1, THI4 and THI5.

Lane 1: YPH500 grown in MM + Thiamine (1.5μM)
Lane 2: YPH500 grown in MM - Thiamine
Lane 3: T48-2D grown in MM + Thiamine (1.5μM)
Lane 4: T48-2D grown in MM - Thiamine
Lane 5: T48-2D(pAN8) grown in MM + Thiamine (1.5μM)
Lane 6: T48-2D(pAN8) grown in MM - Thiamine
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM - Thiamine</td>
<td>MM + Thi (1.5μM)</td>
<td>MM + HMP (2μM)</td>
<td>MM + HET (2μM)</td>
<td>MM + HMP/HET (both 2μM)</td>
</tr>
<tr>
<td>W303a(pUP39a)</td>
<td>1060 ± 138</td>
<td>0.5 ± 0.2</td>
<td>1254 ± 288</td>
<td>1204 ± 249</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>W303a(pRB3)</td>
<td>428 ± 112</td>
<td>0.6 ± 0.2</td>
<td>175 ± 45</td>
<td>299 ± 64</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>W303a(pRB4)</td>
<td>452 ± 113</td>
<td>0.5 ± 0.1</td>
<td>132 ± 20</td>
<td>314 ± 37</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.3 Determining the effect of thiamine precusor addition upon THI4-, THI5- and THI12-lacZ expression

β-galactosidase activities of strains W303a(pUP39a), W303a(pRB3) and W303a(pRB4) after growth in minimal medium containing (i) no additions, (ii) thiamine (1.5μM), (iii) HMP (2μM), (iv) HET (2μM) and (v) HMP and HET (both 2μM). All assays were carried out at least in triplicate on duplicate samples.
**Figure 3.5** Effects of thiamine precursors on *THI4-, THI5- and THI12-lacZ* expression

Graphical representation of the β-galactosidase activity data from Table 3.3
Addition of HMP or HET individually had no repressing effect on THI4-lacZ expression, as mean β-galactosidase activity values for W303a(pUP39a) in the presence of either precursor were slightly greater than those gained in the absence of thiamine. In comparison, THI5-lacZ and THI12-lacZ expression levels were reduced by the addition of HMP or HET to the growth medium. HET caused a 30% decrease and HMP a 60-70% decrease in expression of both reporter genes, compared to the β-galactosidase activity levels gained in the absence of thiamine. When both precursors were added together, all three reporter genes were completely repressed, displaying β-galactosidase activity values of around 1 unit. This result can be explained because upon uptake by the cell, the two precursors will have been converted into TPP causing repression of the reporter genes.

3.4.1 THI5-lacZ and THI12-lacZ expression with increasing HMP concentration

Having found that expression of the THI5-lacZ and THI12-lacZ constructs was reduced by the addition of 2μM HMP, the question arose as to whether further increases in HMP caused further reductions in their expression. β-galactosidase assays were therefore carried out on W303a(pRB3) and W303a(pRB4) grown in medium containing HMP at concentrations of 2μM, 4μM and 10μM (Table 3.4).

Further increases in HMP concentration did not cause any more reduction in β-galactosidase activity; in fact with both reporter constructs, β-galactosidase activities actually rose slightly with the increases in HMP. This implied that addition of HMP does not result in the complete repression of THI5-lacZ and THI12-lacZ expression, as was the case with thiamine.

3.5 Are THI4, THI5 and THI12 dependent upon THI2 and THI3 for expression?

THI2 and THI3 are positively acting regulatory genes that have been shown to be required for the transcriptional activation of all thiamine metabolic genes tested to date (Nosaka et al, 1993). In addition, THI3 is also required for thiamine transport activity. By measuring THI4, THI5 and THI12 expression in the thi2 and thi3 mutant strains O58-M5 and T49-2D, it was possible to determine whether these genes were also dependent upon THI2 and THI3 for activation. Again analysis was carried out using lacZ reporter gene assays and by Northern blot analysis of mRNAs expressed in the mutant strains compared to YPH500. This time however, cultures were grown in medium supplemented with thiamine at concentrations of 0.02μM and 1.5μM, as strains O58-M5 and T49-2D were thiamine auxotrophs. Therefore a concentration of 0.02μM thiamine was used because it allowed the strains to grow, but was sufficiently low to be non-repressing to thiamine gene expression.
Strain p-galactosidase activity in MM + HMP MM + HMP MM + HMP 
(2μM) (4μM) (10μM)

<table>
<thead>
<tr>
<th>Strain</th>
<th>MM + HMP (2μM)</th>
<th>MM + HMP (4μM)</th>
<th>MM + HMP (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pRB3)</td>
<td>175 ± 45</td>
<td>215 ± 62</td>
<td>228 ± 55</td>
</tr>
<tr>
<td>W303a(pRB4)</td>
<td>132 ± 20</td>
<td>158 ± 21</td>
<td>171 ± 46</td>
</tr>
</tbody>
</table>

Table 3.4 *THI5-lacZ* and *THI12-lacZ* expression with increasing HMP concentration

β-galactosidase activity of strains W303a(pRB3) and W303a(pRB4) after growth in medium containing HMP at concentrations of 2μM, 4μM and 10μM. All assays were carried out in triplicate on duplicate samples.
3.5.1 Use of lacZ reporter constructs to monitor the effect of thi2 and thi3 mutations on THI gene expression

Strains O58-M5, T49-2D and YPH500 were transformed with plasmids pUP39a, pRB3 and pRB4, and transformants selected on minimal medium lacking uracil. β-galactosidase assays were then carried out on liquid cultures of the transformants grown in the presence of thiamine (0.02μM and 1.5μM); the results are shown in Table 3.5 and figure 3.6.

Enzyme activity levels measured from all three THI- lacZ reporter constructs were much lower in O58-M5 and T49-2D than in YPH500, under non-repressing conditions of thiamine (0.02μM). The reduction in THI4- lacZ expression was comparable in O58-M5 and T49-2D, with both strains showing around 20 to 30 units of β-galactosidase activity. In the case of the THI5- lacZ and THI12- lacZ constructs, expression was more severely affected in the thi3 mutant strain; both showed less than 1 unit of β-galactosidase activity, and therefore no activation in 0.02μM thiamine. Together these data indicated that THI2 and THI3 are required for the activation of THI4, THI5 and THI12.

3.5.2 Analysis of THI gene expression in the thi2 and thi3 mutant strains by Northern blot hybridisation

To determine whether the chromosomal THI4 and THI5 loci showed reduced expression levels in the thi2 and thi3 mutant strains, Northern blot analysis was carried out on RNAs isolated from O58-M5, T49-2D and YPH500 after growth in medium containing non-repressing (0.02μM) and repressing (1.5μM) levels of thiamine. Identical filters carrying cellular RNAs were hybridised separately with (i) DNA of the ACT1 gene, (ii) the THI4 cDNA, (iii) the THI5 cDNA and (iv) the PHO3 gene. The results of these hybridisations are shown in figures 3.7 and 3.8. Hybridisation with PHO3 acted as a control, as this gene was known to be dependent upon THI2 and THI3 for activation (Nosaka et al, 1993). This was confirmed to be the case, as PHO3 was only weakly expressed in both O58-M5 and T49-2D after growth in the presence of 0.02μM thiamine.

In contrast both THI4 and THI5 were clearly expressed in O58-M5 (thi2) under non-repressing thiamine conditions, although mRNA levels were much reduced in the thi2 mutant strain compared to YPH500 (figure 3.7). In the case of THI4, phosphorimager analysis was carried out to quantitate the level of reduction. By standardising the band intensities via the signal from the ACT1 loading control, it was found that THI4 mRNA levels were reduced by approximately 30% in strain O58-M5 compared to YPH500. These results were not as pronounced as the reporter gene studies (Table 3.5), however they did support the view that THI2 was involved in, but not absolutely required for THI4 and THI5 expression.

55
<table>
<thead>
<tr>
<th>Strain</th>
<th>(\beta)-galactosidase activity in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM + Thi (0.02(\mu)M)</td>
</tr>
<tr>
<td>YPH500(pUP39a)</td>
<td>972 ± 89</td>
</tr>
<tr>
<td>O58-M5(pUP39a)</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>T49-2D(pUP39a)</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>YPH500(pRB3)</td>
<td>422 ± 91</td>
</tr>
<tr>
<td>O58-M5(pRB3)</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>T49-2D(pRB3)</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>YPH500(pRB4)</td>
<td>421 ± 102</td>
</tr>
<tr>
<td>O58-M5(pRB4)</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>T49-2D(pRB4)</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

Table 3.5 THI4-, THI5- and THI12-lacZ expression in thi2 and thi3 mutant strains

\(\beta\)-galactosidase activity in YPH500 (Thi\(^+\)), O58-M5 (thi2) and T49-2D (thi3) transformed with pUP39a, pRB3 and pRB4, after growth in medium containing non-repressing (0.02\(\mu\)M) and repressing (1.5\(\mu\)M) levels of thiamine. All assays were carried out in triplicate on duplicate samples.
Units of β-galactosidase activity in MM + Thi (0.02μM)

Figure 3.6 THI4-, THI5- and THI12-lacZ expression in thi2 and thi3 mutant strains
Graphical representation of the 0.02μM thiamine β-galactosidase activity data from Table 3.5
Figure 3.7 Northern blot analysis of THI4 and THI5 expression in a thi2 mutant strain

Total cellular RNAs from strains YPH500 (THI2) and O58-M5 (thi2) grown in non-repressing (0.02µM) and repressing (1.5µM) levels of thiamine, were hybridised with probes specific to ACT1, THI4, THI5 and PHO3

Lane 1: YPH500 grown in MM + Thiamine (1.5µM)
Lane 2: YPH500 grown in MM + Thiamine (0.02µM)
Lane 3: O58-M5 grown in MM + Thiamine (1.5µM)
Lane 4: O58-M5 grown in MM + Thiamine (0.02µM)
Figure 3.8 Northern blot analysis of THI4 and THI5 expression in a thi3 mutant strain
Total cellular RNAs from strains YPH500 (THI3) and T49-2D (thi3) grown in non-repressing (0.02µM) and repressing (1.5µM) levels of thiamine, were hybridised with probes specific to ACT1, THI4, THI5 and PHO3
Lanes 1 and 2: YPH500 grown in MM + Thiamine (0.02µM)
Lanes 3 and 4: T49-2D grown in MM + Thiamine (0.02µM)
Lanes 5 and 6: YPH500 grown in MM + Thiamine (1.5µM)
Lanes 7 and 8: T49-2D grown in MM + Thiamine (1.5µM)
In the Northern blot analysis of the T49-2D (thi3) sample, a band of reduced intensity and increased smearing was observed for ACT1 mRNA in T49-2D grown with 0.02µM thiamine (Lanes 3 and 4, figure 3.8). This was not due to a reduction in the amount of RNA used, but was caused by the very slow growth of this strain under limiting thiamine conditions. RNA of poor quality that showed extensive degradation, even when freshly made, was always obtained from T49-2D grown in minimal medium containing 0.02µM thiamine, compared to 1.5µM thiamine. Despite this degradation, by using the intensities of the ACT1 hybridisation results as a standardisation control, it was still possible to determine that the THI4 and THI5 mRNA levels were reduced in T49-2D. These data therefore supported the β-galactosidase activity results (Table 3.5), and proved that THI4, THI5 and THI12 were also dependent upon THI3 for complete transcriptional activation when TPP levels were not repressing.

3.6 Discussion

Using a PCR-based approach, promoter-lacZ reporter gene constructs were made for two members of the THI5 gene family, these being THI5-lacZ and THI12-lacZ. Subsequent β-galactosidase assays on strains carrying these constructs showed that both promoters are expressed to the same high level in the absence of thiamine, and completely repressed in the presence of thiamine. This implies that the promoter of the THI12 chromosomal locus is also functional, and is the first direct evidence that a gene other than THI5 from within this gene family is transcriptionally active. It will therefore be of interest to construct similar reporter fusions specific for the THI11 and THI13 gene promoters, to determine whether they too are expressed, and if so to what extent. If they are expressed, it will be of interest to see if expression levels from a given THI5-like gene reporter construct are affected by stepwise deletions of the three remaining gene family members. One possible result is that expression from the reporter gene will increase with each gene deletion, as the cell attempts to maintain a constant cumulative level of expression from the four loci.

The THI4-, THI5- and THI12-lacZ constructs all displayed derepressed expression in the thi80 mutant T48-2D, indicating that like all previous thiamine genes tested, they too are regulated in response to intracellular TPP levels (Nishimura et al, 1991). The extent of derepression for the three genes was not constant, suggesting that the different genes possess different TPP thresholds at which repression no longer occurs. However a second possibility arises from the finding that THI5- and THI12-lacZ expression is also repressed by precursor concentrations, most noticeably HMP. Regulation of thiamine genes by HMP has also been shown for the THI5 homologue in S.pombe, nmt1, although it has not
been reported whether repression is as great as the 60% reduction in β-galactosidase activity observed here (Zurlinden and Schweingruber, 1994).

Under derepressed conditions expression of THI4-, THI5- and THI12-lacZ was found to be dependent upon the transcriptional activator proteins Thi2 and Thi3. All three promoters displayed greatly reduced expression in the thi2 and thi3 mutant strains compared to the wild-type strain YPH500, in medium containing 0.02μM thiamine. With the exception of THI4 expression in O58-M5, the β-galactosidase data was clearly supported by Northern blot analysis of the corresponding chromosomal loci; these too showed reduced transcriptional activity under non-repressing conditions. Although phosphorimager analysis did show that THI4 mRNA expression was reduced by approximately 30% in O58-M5, this decrease was nowhere near as great as the observed drop in β-galactosidase activity, from 1000 to 30 units, seen with the THI4-lacZ reporter gene.

It is unlikely that Thi2p, a Cys6 zinc finger containing protein that has been shown to be involved in the transcriptional regulation of PHO3 and THI5, could also have a role in the translational regulation of THI4. More feasible possibilities for detecting a greater reduction in THI4-lacZ enzyme activity than THI4 mRNA levels in O58-M5, are related to mRNA turnover. As each mRNA molecule will be translated into many primary polypeptide molecules, any decreases in transcription would be amplified after translation. Recently a fully functional THI4 gene tagged with c-myc has become available, on the plasmid pUR2016 supplied by A. Mooren, Unilever. Using this construct, it will now be possible to compare directly levels of THI4 mRNA and protein within mutant and wild-type cells after growth under non-repressing conditions of thiamine. This experiment will therefore provide a more accurate way of measuring Thi4p production in the thi2 mutant strain O58-M5.

Additional work in this laboratory has uncovered a third gene, PDC2, involved in the positive regulation of thiamine genes, including THI4, THI5 and THI12 (Richards, 1996). It will therefore be of interest to determine whether expression levels of THI4 are reduced further in strains that contain double or even triple combinations of the thi2, thi3 and pdc2 mutations. This analysis could again be carried out using lacZ-reporter gene based assays, Northern blot assays, and Western blot assays on the c-myc tagged Thi4p.
CHAPTER FOUR
ISOLATION AND GENETIC CHARACTERIZATION OF MUTANTS WITH DEREPRESSED THI4-lacZ ACTIVITY

4.1 Introduction

In *S. cerevisiae* three genes, THI2, THI3 and PDC2 are known to function in the transcriptional activation of thiamine metabolic genes (Kawasaki *et al.*, 1990; Nishimura *et al.*, 1992b; Richards, 1996). In contrast no genes have been described that act to repress or deactivate this expression in response to a rise in intracellular levels of the negative effector TPP. The principal aim of this project was therefore to isolate mutant strains of *S. cerevisiae* displaying derepressed expression of the thiamine biosynthetic gene THI4 in the presence of thiamine. Such mutations could define the negatively acting components of the THI4 transcriptional control system.

A similar study has already been carried out in the fission yeast *S. pombe*, with three classes of mutants being identified, tnr1, tnr2 and tnr3, that gave rise to constitutive expression of the genes nmt1 and pho4 (Schweingruber *et al.*, 1991). The mutant tnr3 has been shown to affect thiamine pyrophosphokinase, and is equivalent to the *S. cerevisiae* thi80 mutation (Fankhauser *et al.*, 1995). The remaining two mutants however are as yet uncharacterized, and may represent new genes concerned with the negative regulation of thiamine metabolism.

4.2 Isolation of derepresed THI4-lacZ mutant strains

4.2.1 Development of a suitable screen for mutagenesis

In order to isolate mutants defective in the transcriptional control of THI4 expression, an appropriate screen was required for *S. cerevisiae* grown on solid medium. It had been shown that the THI4-lacZ reporter gene on pUP39a enabled THI4 expression to be monitored by assaying β-galactosidase activity in liquid grown cultures (Praekelt *et al.*, 1994), however no such studies had been carried out on cells grown on solid medium. This type of screen would require a synthetic minimal medium base so that thiamine concentrations could be accurately controlled, X-gal to provide a chromogenic substrate for the β-galactosidase, and buffering at pH 6.5 - 7.0 to prevent enzyme denaturation (Clifton *et al.*, 1978).

The *S. cerevisiae* strain W303a was therefore transformed with pUP39a and grown on M63 based minimal medium containing X-gal, in the presence and absence of thiamine; a thiamine concentration of 1.5μM was chosen as this was known to completely repress THI4 expression throughout the growth of batch cultures. The results of this growth test are shown in Table 4.1 and in Figure 4.1. As expected only in the absence of thiamine was the reporter gene expressed, resulting in the hydrolysis of X-gal by β-galactosidase, and the release of the blue coloured compound 5-Bromo-4-chloro-3-indigo. The colony colour observed was independent
<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Colony colour</th>
<th>Reporter gene status</th>
</tr>
</thead>
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<td>W303a(pUP39a)</td>
<td>X-gal - Thiamine</td>
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</tr>
<tr>
<td>W303a(pUP39a)</td>
<td>X-gal + Thi (1.5(\mu)M)</td>
<td>White</td>
<td>Reporter gene OFF</td>
</tr>
</tbody>
</table>

Table 4.1 W303a(pUP39a) colony colour on X-gal medium
W303a(pUP39a) was assayed for \(THI4-lacZ\) expression by colony colour on X-gal medium in the presence (1.5\(\mu\)M) and absence of thiamine.

Figure 4.1 W303a(pUP39a) grown on X-gal medium
of the reporter gene status prior to plating onto the X-gal medium, as the same result was seen whether W303a(pUP39a) had been grown previously on medium containing or lacking thiamine. Although the blue colouration of colonies on a given X-gal plate lacking thiamine were not uniform, no colonies showed any signs of 'blueness' in the presence of thiamine. This meant that it should be possible to select for mutants displaying derepressed expression of the reporter gene, by screening for blue colonies on X-gal medium containing thiamine (1.5μM).

4.2.2 Mutagenesis of W303a(pUP39a)

To generate a diverse range of mutations within strain W303a(pUP39a), it was decided to use UV light as a mutagen. UV light is an efficient mutagen causing a variety of substitutions including transitions, transversions, and frame shifts, in particular those of a single nucleotide deletion (Kunz et al, 1987 and Lee et al, 1988). A survival curve was produced for the treatment of W303a(pUP39a) with UV, and the mutagen dosage causing 50% cell survival calculated to be 0.5 Jm⁻²s⁻¹ for 150 seconds.

Approximately 17,000 colonies from UV treated cells were screened on X-gal medium containing thiamine (1.5μM), for any that were blue in colouration; 69 possibles were picked, restreaked onto minimal medium lacking uracil to generate a stock plate of each, and then retested on X-gal medium. Of those tested, 37 reproducibly gave blue colonies and so were taken on for further analysis. The mutants were termed det, standing for derepressed expression of THI4-lacZ in the presence of thiamine.

4.2.3 Assays of THI4-lacZ expression in liquid cultures

β-galactosidase assays were carried out on liquid cultures of the 37 mutants to check that the blue colouration was indeed due to aberrant THI4-lacZ expression. In addition this enabled me to quantitate their level of derepression. Table 4.2 shows the ten det(pUP39a) mutants which gave the greatest levels of β-galactosidase activity in the presence of thiamine (1.5μM). Two of the mutants, det1(pUP39a) and det2(pUP39a), looked particularly interesting giving high activity values. In the case of det1, the levels of β-galactosidase activity were comparable to those of the wild-type strain grown in the absence of thiamine. Of the remaining 27 tested, 21 gave expression levels higher than the repressed wild-type strain. However in these cases levels of β-galactosidase activity were less than two units and therefore considered too low to work with. The final six mutants gave β-galactosidase expression levels less than or equal to those from a fully repressed THI4-lacZ construct in a non-mutagenized wild-type strain, and so were also discarded.
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in</th>
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<tbody>
<tr>
<td></td>
<td>MM + Thi (1.5μM)</td>
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<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>det1(pUP39a)</td>
<td>752</td>
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<tr>
<td>det2(pUP39a)</td>
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<td>det3(pUP39a)</td>
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<td>det4(pUP39a)</td>
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<td>det6(pUP39a)</td>
<td>5.7</td>
</tr>
<tr>
<td>det7(pUP39a)</td>
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</tr>
<tr>
<td>det8(pUP39a)</td>
<td>3.6</td>
</tr>
<tr>
<td>det9(pUP39a)</td>
<td>3.2</td>
</tr>
<tr>
<td>det10(pUP39a)</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 4.2 THI4-lacZ expression in det mutants 1 to 10(pUP39a)

β-galactosidase activities of the ten det(pUP39a) mutants showing the highest level of derepressed THI4-lacZ expression. Assays were carried out in the presence of thiamine (1.5μM), compared to wild-type W303a(pUP39a) grown in the presence and absence of thiamine. All assays on det(pUP39a) mutants were carried out on duplicate samples. nt indicates not tested.
4.2.4 Analysis of whether the mutations are plasmid or chromosomal in origin

As the mutagenesis screen involved the assay of a plasmid-based reporter gene, it was possible that the mutations resulting in derepressed $THI4$-$lacZ$ expression were either plasmid or chromosomal in origin. The following strategies were therefore used to determine the nature of each mutation:

(i) Plasmid pUP39a was recovered from each of the ten mutants into $E.coli$, purified and transformed into wild-type W303a. Transformants were then assayed for $\beta$-galactosidase activity in the presence of thiamine; if reporter gene expression was still derepressed, this would imply a plasmid-based mutation.

(ii) Derivatives of the mutants that had lost pUP39a were isolated, retransformed with wild-type pUP39a and then assayed for $\beta$-galactosidase activity in the presence of thiamine. In this case if the cultures showed a derepressed phenotype, it would imply that the mutation was chromosomal in origin.

4.2.4.1 Investigation of plasmid-based mutations

Plasmid DNA was recovered from each of the mutant strains and transformed into the $E.coli$ strain XL1-Blue. The amplified plasmid DNA was then purified from $E.coli$, a sample digested with the restriction enzyme $BamHI$ to check it was pUP39a, prior to being transformed into wild-type W303a. In all ten cases, strain W303a transformed with rescued pUP39a plasmid DNA showed wild-type repressed levels of $THI4$-$lacZ$ expression in the presence of thiamine. Therefore the derepressed expression phenotype of $det$ mutants 1 to 20(pUP39a) were not the result of plasmid-based mutations.

4.2.4.2 Investigation of chromosomal mutations

The $det$ mutants 1 to 10(pUP39a) were allowed to lose the $URA3$ plasmid pUP39a by growth on non-selective uracil-containing medium, as described in the experimental procedures. The $ura3$ plasmid-free mutant strains were then transformed with wild-type pUP39a, uracil prototrophs selected, and retested for $THI4$-$lacZ$ expression in the presence of thiamine (Table 4.3). Consistent with the results in section 4.2.4.1, all ten pUP39a retransformed $det$ strains still displayed the derepressed $THI4$-$lacZ$ expression phenotype, and therefore contained chromosomal-based mutations.

4.3 Genetic analysis of $det$ mutants 1 to 10

Having isolated ten $det$ mutant strains, it was then important to carry out a genetic characterization of them. This involved analysis as to whether:

(i) the phenotype arose as a result of dominant or recessive mutations.

(ii) each $det$ strain resulted from a mutation in a single gene or, as could be the case with a random mutagenesis approach, was due to mutations in more than one gene.
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
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<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
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<td>det1(pUP39a)</td>
<td>846 ± 45</td>
</tr>
<tr>
<td>det2(pUP39a)</td>
<td>247 ± 56</td>
</tr>
<tr>
<td>det3(pUP39a)</td>
<td>10 ± 2.5</td>
</tr>
<tr>
<td>det4(pUP39a)</td>
<td>6.6 ± 0.8</td>
</tr>
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<td>det5(pUP39a)</td>
<td>4.7 ± 1.6</td>
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<tr>
<td>det6(pUP39a)</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>det7(pUP39a)</td>
<td>5.3 ± 0.6</td>
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<tr>
<td>det8(pUP39a)</td>
<td>4.5 ± 1.7</td>
</tr>
<tr>
<td>det9(pUP39a)</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>det10(pUP39a)</td>
<td>2.1 ± 0.7</td>
</tr>
</tbody>
</table>

Table 4.3 THI4-lacZ expression in "cured" det mutants 1 to 10 retransformed with wild-type pUP39a
β-galactosidase activities of det1 to det10 retransformed with wild-type pUP39a, grown in the presence of thiamine (1.5μM). All assays were carried out at least in triplicate on duplicate samples.
the number of complementation groups and therefore genes that were represented by the ten mutant strains.

4.3.1 Dominance tests

To test whether the mutants det1 to 10 carried dominant or recessive mutations, each mutant was crossed with the wild-type strain KBY4 to produce heterozygous diploids. As a control, the parental strain W303a(pUP39a) was also crossed with strain KBY4. KBY4 is isogenic to W303α except for it being HIS3 (K. Byrne, pers. comm.). Therefore the resulting diploids were selected on minimal medium lacking uracil and histidine. The DET/DET homozygous diploid was termed CD1(pUP39a) and the det/DET heterozygous diploids produced from each of the mutants det1 to 10(pUP39a) crossed with KBY4, called RHD1 to 10(pUP39a) respectively.

β-galactosidase assays were then carried out on RHD1 to 10(pUP39a) grown in the presence of thiamine (1.5μM), and compared to CD1(pUP39a) grown in the presence and absence of thiamine (Table 4.4). As expected the control diploid strain CD1(pUP39a) showed repression of THI4-lacZ expression by exogenous thiamine, although activity levels in the absence of thiamine were consistently lower than those of W303a(pUP39a). All heterozygous diploid strains except for RHD1(pUP39a) gave very low β-galactosidase activities, less than 1 unit, in the presence of thiamine. These values were effectively the same as the activity levels measured in both the wild-type haploid and diploid control strains in the presence of thiamine, indicating that det mutants 2 to 10 carried recessive mutations.

Strain RHD1(pUP39a) was unusual in that it exhibited approximately 28 units of β-galactosidase activity in the presence of thiamine, a clear THI4-lacZ derepressed phenotype. However this level of derepression was nowhere near as great as that of its haploid counterpart det1(pUP39a), which gave β-galactosidase activities in excess of 800 units under the same conditions. It therefore appeared that det1 was caused by neither a completely dominant nor completely recessive mutation, but was instead the result of a partially dominant or semi-dominant mutation.

4.4 Isolation of dominant mutations using a diploid strain

In view of the finding that det1 was caused by a partially dominant mutation, it was decided to mutagenize a diploid strain containing pUP39a, in an attempt to isolate det mutant strains caused by completely dominant mutations. A survival curve of the diploid strain CD1(pUP39a) treated with UV was constructed, and again a dosage of 0.5 Jm⁻²s⁻¹ for 150 seconds resulted in 50% cell survival.

Approximately 30,000 colonies from UV treated CD1(pUP39a) cells were screened on medium containing X-gal and thiamine (1.5μM), and four blue coloured colonies were detected. These colonies were restreaked onto minimal medium
Strain p-galactosidase activity in
MM + Thi (1.5\mu M) MM - Thiamine

<table>
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<tr>
<th>Strain</th>
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<th>MM + Thi (1.5\mu M)</th>
<th>MM - Thiamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
<td>1060 ± 138</td>
<td></td>
</tr>
<tr>
<td>CD1(pUP39a)</td>
<td>0.5 ± 0.3</td>
<td>804 ± 108</td>
<td></td>
</tr>
<tr>
<td>RHD1(pUP39a)</td>
<td>28 ± 2.8</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD2(pUP39a)</td>
<td>0.8 ± 0.1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD3(pUP39a)</td>
<td>0.5 ± 0.1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD4(pUP39a)</td>
<td>0.6 ± 0.1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD5(pUP39a)</td>
<td>0.4 ± 0.1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD6(pUP39a)</td>
<td>0.3 ± 0.1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD7(pUP39a)</td>
<td>0.2 ± 0.1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RHD8(pUP39a)</td>
<td>0.4 ± 0.1</td>
<td>nt</td>
<td></td>
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<tr>
<td>RHD9(pUP39a)</td>
<td>0.3 ± 0.1</td>
<td>nt</td>
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</tr>
<tr>
<td>RHD10(pUP39a)</td>
<td>0.5 ± 0.2</td>
<td>nt</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4 *THI4-lacZ* expression in det/DET heterozygous diploid strains RHD1 to RHD10(pUP39a)

β-galactosidase activities of strains RHD1 to RHD10(pUP39a) in the presence of thiamine (1.5\mu M), compared to the wild-type control strains W303a(pUP39a) and CD1(pUP39a) in the presence and absence of thiamine. All assays were carried out at least in triplicate on duplicate samples. nt indicates not tested.
selective for the diploid strain, i.e. lacking uracil and histidine, prior to retesting colony colouration on X-gal medium. Three strains still gave rise to blue colonies and so were assayed for β-galactosidase activity in liquid culture (Table 4.5). The three mutants, named det11, det12 and det13(pUP39a), displayed THI4-lacZ expression in the presence of thiamine; levels of derepression ranged from 20 to 66 units. These values were similar to the 28 units recorded for the heterozygous diploid RHD1(pUP39a), therefore making it of interest to see what THI4-lacZ expression levels these mutants produced when in the haploid state.

4.4.1 Analysis of whether det mutants 11, 12 and 13(pUP39a) are chromosomal or plasmid in origin

As in the haploid screen (section 4.2.4.1), the pUP39a plasmid DNAs recovered from det mutants 11 to 13(pUP39a) failed to confer derepressed THI4-lacZ expression phenotypes when transformed into a wild-type strain, in this case the diploid CD1. Mutants det11, det12 and det13 were therefore not plasmid-based mutations, but instead must also be due to chromosomal-based mutations. This was the case as all three det strains cured of pUP39a and retransformed with wild-type pUP39a, still displayed derepressed THI4-lacZ expression in the presence of thiamine (1.5μM). These data are shown in Table 4.6. Further analysis of the mutants det11, det12 and det13 was carried out in conjunction with the heterozygous diploid strains RHD1 to 10(pUP39a).

4.5 Meiotic segregation analysis

To determine whether det mutants 1 to 13 were the result of mutations within single or multiple genes, the segregation pattern of the det phenotype was examined in haploid progeny resulting from meiosis of det/DET heterozygous diploids. A segregation pattern of 2 : 2 for DET : det within all the tetrads analysed for a given mutant would indicate a mutation within a single nuclear gene (Sherman and Hicks, 1991). Any deviation from this segregation pattern, i.e. the occurrence of 3 : 1 or 4 : 0 ratios, would imply mutations in more than one gene. Diploid strains CD1(pUP39a), RHD1 to 10(pUP39a) and det11 to 23(pUP39a) were therefore induced to undergo meiosis, and the production of meiotic tetrads monitored microscopically. The products of meiosis (4 spores) within each ascus were separated using a Singer MSM Micromanipulator and the segregation pattern of pairs of alleles at the HIS3, URA3 and DET loci analysed for at least five complete tetrads from each diploid. As all the diploids being tested were HIS3/his3, segregation of this chromosomal mutation acted as an internal control; if 2 : 2 segregation for HIS+:his− was detected then the four spores were considered to constitute a genuine tetrad. Segregation of the URA3 allele was also studied to identify those spores that did not receive a copy of the plasmid pUP39a following meiosis. These ura− meiotic segregants then had to be
Strain P-galactosidase activity in MM + Thi (1.5μM)

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1(pUP39a)</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>det11(pUP39a)</td>
<td>20</td>
</tr>
<tr>
<td>det12(pUP39a)</td>
<td>35</td>
</tr>
<tr>
<td>det13(pUP39a)</td>
<td>66</td>
</tr>
</tbody>
</table>

Table 4.5 THI4-lacZ expression in diploid mutant strains det11, det12 and det13
β-galactosidase activities of det11 to det13(pUP39a) compared to CD1(pUP39a) after growth in the presence of thiamine (1.5μM). Assays on det mutants were carried out on duplicate samples.

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1(pUP39a)</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>det11(pUP39a)</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>det12(pUP39a)</td>
<td>34 ± 9</td>
</tr>
<tr>
<td>det13(pUP39a)</td>
<td>55 ± 16</td>
</tr>
</tbody>
</table>

Table 4.6 THI4-lacZ expression in det11 to det13 containing wild-type pUP39a
β-galactosidase activities of det11 to 13 retransformed with wild-type pUP39a after growth in the presence of thiamine (1.5μM). All assays were carried out at least in triplicate on duplicate samples.
retransformed with pUP39a prior to testing the segregation of \( \text{DET} : \text{det} \) within the meiotic progeny. Table 4.7 summarises the \( \text{HIS3} \) and \( \text{DET} \) segregation analysis, and Table 4.8 gives \( \beta \)-galactosidase activity data for an example tetrad from each of the diploid strains that sporulated successfully with a 2 : 2 segregation pattern for \( \text{HIS}^+ : \text{his}^- \).

As expected the control diploid strain CD1 produced tetrads displaying a segregation pattern of 2 : 2 for \( \text{HIS}^+ : \text{his}^- \), and 4 : 0 for \( \text{DET} : \text{det} \), i.e. all meiotic segregants displayed wild-type repression of \( \text{THI4-lacZ} \) by exogenous thiamine. Four of the heterozygous diploid strains, RHD3, RHD4, RHD7 and RHD10 failed to produce tetrads when grown on either solid or liquid sporulation medium. This failure to sporulate must have been due to an additional mutation(s) present within the haploid \( \text{det} \) mutants 3, 4, 7 and 10 that was gained during UV mutagenesis. To see if an alternative \( \text{DET} \) wild-type strain could rescue this sporulation defect, each mutant was crossed with strain DBY746 and the resulting heterozygous diploids grown on solid and liquid sporulation medium. Again no tetrads were produced, therefore no further analysis was carried out on these mutants.

The remaining nine heterozygous diploid strains gave tetrads showing the expected 2 : 2 segregation pattern for \( \text{HIS}^+ : \text{his}^- \), except for \( \text{det11(pUP39a)} \), which consistently gave 4 : 0 segregation of \( \text{HIS}^+ : \text{his}^- \) in 15 tetrads tested. The mutant \( \text{det11(pUP39a)} \) was isolated after UV mutagenesis of the diploid CD1(pUP39a). It was therefore possible that \( \text{det11(pUP39a)} \) had become homozygous for \( \text{HIS3} \) either as a result of the mutagenesis or through spontaneous reversion of the \( \text{his3} \) point mutation. Initial analysis of \( \text{DET} \) segregation in this mutant was also unusual, in that a 3 : 1 pattern for white : blue colonies was seen on medium containing X-gal and thiamine (1.5\( \mu \)M) for the first four tetrads tested; a ratio implying that the derepressed \( \text{THI4-lacZ} \) phenotype of \( \text{det11} \) was the result of mutations in more than one gene. Taking these segregation data together, it was decided to leave \( \text{det11(pUP39a)} \) and concentrate upon the remaining eight mutant strains that gave the 2 : 2 segregation pattern of wild-type : mutant phenotype for both the \( \text{HIS3} \) and \( \text{DET} \) loci.

The two \( \text{det} \) meiotic segregants in each tetrad from diploids RHD1 and RHD2(pUP39a) (shown in bold type in Table 4.8), gave \( \text{THI4-lacZ} \) expression values greater than those of the original \( \text{det1} \) and \( \text{det2(pUP39a)} \) haploid mutants. This was particularly noticeable for RHD2(pUP39a) which produced \( \text{det} \) progeny with an average \( \beta \)-galactosidase activity value of 711 ± 151 in the presence of thiamine (1.5\( \mu \)M), compared to the 195 units measured in the original mutant \( \text{det2(pUP39a)} \). This increase in expression levels must have been due to the healthier genetic background of the RHD1 and RHD2 segregants resulting from crossing each mutant with the wild-type strain KBY4.

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Tetrads produced?</th>
<th>Segregation of (HIS3:his3)</th>
<th>Segregation of (DET:det)</th>
<th>Single gene mutant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>4:0</td>
<td>---</td>
</tr>
<tr>
<td>RHD1(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>RHD2(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>RHD3(pUP39a)</td>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RHD4(pUP39a)</td>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RHD5(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>RHD6(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>RHD7(pUP39a)</td>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RHD8(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>RHD9(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>RHD10(pUP39a)</td>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(det11)(pUP39a)</td>
<td>Yes</td>
<td>4:0</td>
<td>3:1</td>
<td>No</td>
</tr>
<tr>
<td>(det12)(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
<tr>
<td>(det13)(pUP39a)</td>
<td>Yes</td>
<td>2:2</td>
<td>2:2</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Table 4.7** \(HIS3\) and \(DET\) segregation following meiosis

Table showing the meiotic segregation patterns of \(HIS3\) and \(DET\) in the diploid strains CD1, RHD1 to RHD10 and \(det11\) to \(det13\)
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-gal activity in MM + Thi (1.5μM) for meiotic segregant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>CD1(pUP39a)</td>
<td>0.5</td>
</tr>
<tr>
<td>RHD1(pUP39a)</td>
<td>999</td>
</tr>
<tr>
<td>RHD2(pUP39a)</td>
<td>783</td>
</tr>
<tr>
<td>RHD5(pUP39a)</td>
<td>7.3</td>
</tr>
<tr>
<td>RHD6(pUP39a)</td>
<td>6.6</td>
</tr>
<tr>
<td>RHD8(pUP39a)</td>
<td>5.9</td>
</tr>
<tr>
<td>RHD9(pUP39a)</td>
<td>2.6</td>
</tr>
<tr>
<td>det12(pUP39a)</td>
<td>571</td>
</tr>
<tr>
<td>det13(pUP39a)</td>
<td>1377</td>
</tr>
</tbody>
</table>

Table 4.8 THI4-lacZ expression in the products of meiosis

β-galactosidase activities of the four meiotic segregants from an example tetrad from each of the diploid strains CD1(pUP39a), RHD1, RHD2, RHD5, RHD6, RHD8 and RHD9(pUP39a), and det12 and det13(pUP39a) Assays were carried out after growth in medium containing thiamine (1.5μM), and those segregants displaying derepressed THI4-lacZ expression are shown in bold type. Assays were carried out on duplicate samples.
Of particular interest was the $THI4$-lacZ expression levels of the $det$ meiotic segregants which arose from diploids $det12$ and $det13$ (pUP39a), as these two mutant strains were isolated by mutagenesis of the diploid CD1 (pUP39a). In each case the $det$ haploid segregants showed $\beta$-galactosidase activity levels far greater than the 35 and 66 units recorded for $det12$ and $det13$ (pUP39a) diploids respectively. This result indicated that the $det12$ and $det13$ mutations behaved in a similar way to the $det1$ mutation, in that there was a reduced mutant phenotype in the heterozygous diploid state compared to the haploid state. These two mutant strains were therefore also the result of partially dominant mutations. Also as $det12$ and $det13$ showed $2:2$ segregation of the $DET:det$ phenotypes, the original diploids isolated must have been heterozygous for the respective $det$ alleles. In accordance with earlier nomenclature, the original diploids should therefore have been referred to as RHD12 and RHD13, with the mutant haploid progeny from these diploids being called $det12$ and $det13$.

The final four strains, RHD5, RHD6, RHD8 and RHD9, produced $det$ meiotic segregants with $\beta$-galactosidase activity values less than ten units, which were comparable to the derepressed activities recorded in the original haploid mutants.

4.6 Complementation analysis

Thus eight strains were isolated that each displayed a $det$ phenotype due to a mutation within a single nuclear gene. Complementation analysis was carried out on these strains to ascertain the number of different mutant genes they represented. However this analysis was complicated because three of the strains were the result of partially dominant mutations, while the other five carried recessive mutations. In addition four of the five recessive mutant strains displayed a low level derepressed phenotype, characterized by $\beta$-galactosidase activities greater than two but less than ten units. The fifth recessive mutation, $det2$ (pUP39a) gave a $\beta$-galactosidase activity level of 700 units, a phenotype more similar to that of the three partial dominant mutations in the haploid state. I therefore decided to divide the eight strains into two groups of four according to their respective levels of $THI4$-lacZ reporter gene derepression and analyse them separately. One group comprised $det5$, $det6$, $det8$ and $det9$; the other group comprised $det1$, $det2$, $det12$ and $det13$.

4.6.1 Complementation analysis of $det$ mutations 5, 6, 8 and 9

Complementation tests on $det$ mutations 5, 6, 8 and 9, required the construction of heterozygous diploids between all mutant strains and homozygous diploids for each mutation. This was achieved by carrying out matings of the type shown overpage, with diploids being selected on the basis of histidine and uracil prototrophy:-
The original \textit{det} mutants 5, 6, 8 and 9(pUP39a) all possessed the phenotype \textit{his}$^{-}$, \textit{URA}$^{+}$ (mutant type 1 in the cross above), therefore only variants of each mutant strain with the genotype of mutant type 2 needed to be found. This was achieved by screening for meiotic segregants of the diploid strains RHD5, RHD6, RHD8 and 9(pUP39a) (section 4.5), that possessed the appropriate \textit{HIS}$^{+}$, \textit{ura}$^{-}$ phenotype. The diploids resulting from these crosses were then assayed for \textit{THI4-lacZ} expression in the presence of thiamine (1.5\textmu M), and compared to \beta-galactosidase levels recorded from the \textit{DET/DET} homozygous diploid CD1(pUP39a). Data are recorded in Table 4.9.

All heterozygous diploids formed by the mating of two different mutant strains gave wild-type repressed levels of \beta-galactosidase activity in the presence of thiamine. Normally this would indicate that complementation had occurred, and that the \textit{det} mutant strains 5, 6, 8 and 9 possessed mutations within four separate genes. However this may not be the case, as all four homozygous mutant diploid strains (shown in bold type in Table 4.9) also gave wild-type repressed \beta-galactosidase activities! This result made any conclusions regarding complementation within the heterozygous diploids impossible.

4.6.2 Complementation analysis of \textit{det} mutations 1, 2, 12 and 13

As \textit{det}1, \textit{det}12 and \textit{det}13 contained partially dominant mutations, the method of complementation analysis used for \textit{det} strains 5, 6, 8 and 9, had to be slightly modified. Instead of determining whether diploids formed between two haploid \textit{det} mutants displayed the \textit{det} mutant phenotype, I looked at segregation of the \textit{det} phenotype into the products of meiosis from these diploids. The principle behind this was that allelic mutations should segregate 4 : 0 for the mutant (\textit{det}) : wild-type (\textit{DET}) phenotype, whereas mutations at different loci would generate recombinant \textit{DET}$^{+}$ progeny; recombination between two different mutations within the same gene would be a relatively rare event.

Heterozygous and homozygous diploids were therefore established by crossing each \textit{det} strain against itself and the three other \textit{det} mutant strains. This was achieved by carrying out matings of the type shown below, with diploids being selected on the basis of histidine and uracil prototrophy. Again haploid \textit{det} mutants with the genotype of mutant 2 were gained from the meiotic segregants of diploids RHD1, RHD2, RHD12 and RHD13 (section 4.5).
Table 4.9 Complementation analysis of *det* mutations 5, 6, 8 and 9

Table showing the crosses set up between the different mutant strains, the resulting diploids and the β-galactosidase activities in the presence of thiamine (1.5µM). Homozygous diploids for each mutation are shown in bold type, and data from CD1(pUP39a) are included as a control. Assays were carried out twice on duplicate samples.
These diploids were induced to undergo meiosis and the meiotic products from a number of four spore asci separated using a Singer MSM micromanipulator. To test that the four spores did arise from genuine tetrads, segregation of the HIS3 and his3 alleles was again analysed. Tetrads that showed 2 : 2 segregation for HIS+ : his- were then studied for segregation of the det mutant phenotype, by measuring the β-galactosidase activity of each meiotic product grown in the presence of thiamine (1.5μM). Table 4.10 shows the various matings carried out, the diploids formed and their segregation patterns for the det mutant phenotype.

As expected the four homozygous diploid strains (shown in bold type in Table 4.10), gave a 4 : 0 segregation pattern for det : DET. Of the diploids formed by crossing together two different det strains, all except D2X13 gave a variety of segregation ratios for det : DET in the tetrads studied. These diploids were therefore formed from haploid mutants containing det mutations within different genes, whereas the mutations det2 and det13 must be allelic. The mutant strains det1, det2, det12 and det13, therefore represent mutations within three different genes, and so will be renamed det1, det2-2, det12 and det2-13.

4.7 β-galactosidase assays on homozygous diploids

During the complementation analysis of det mutations 5, 6, 8 and 9, it was found that their respective homozygous diploid strains displayed wild-type levels of THI4-lacZ expression in the presence of thiamine. It was therefore of interest to see how the levels of β-galactosidase activity in homozygous mutant diploid strains D1X1, D2X2, D12X12, and D13X13(pUP39a), compared to those of their haploid counterparts. The data from this analysis were also used to check further whether mutants det1, det12 and det2-13 were the result of partially or completely dominant mutations. This was achieved by comparing the levels of β-galactosidase activity in the homozygous diploid strains to those of the corresponding heterozygous mutant diploid strains. Thus assays were carried out to measure THI4-lacZ expression in the pUP39a containing haploid det mutants 1, 2-2, 12, 2-13, heterozygous diploids RHD1, RHD2, RHD12, RHD13, and the homozygous diploids D1X1, D2X2, D12X12, and D13X13 in medium containing thiamine (1.5μM).

It was clear from Table 4.11 and figure 4.2 that β-galactosidase activities detected in the homozygous diploid mutants were consistently lower than those recorded from the haploid mutant strains. This large decrease in detectable β-galactosidase activity between det mutants in the haploid and diploid states may explain why those det mutant strains displaying only two to ten units of β-galactosidase activity when haploid were not distinct from wild-type strains when diploid. The results in figure 4.2 also supported the conclusion that det1, det12 and det2-13 were the result of partially dominant and not completely dominant mutations. This was because the diploid strains heterozygous for each of the three
### Table 4.10 Complementation analysis of det mutations 1, 2, 12 and 13

Table showing the crosses set up between the different mutant strains, and the segregation pattern of the det mutant phenotype within the tetrads of the resulting diploid strains.

<table>
<thead>
<tr>
<th>Mutant strains crossed</th>
<th>Diploid</th>
<th>Number of tetrads with det : DET segregation pattern of</th>
<th>Homozygous det Diploid? (Allelic?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>det1(pUP39a) x det1</td>
<td>D1X1</td>
<td>9 : 0 : 0 : 0</td>
<td>Yes</td>
</tr>
<tr>
<td>det1(pUP39a) x det2</td>
<td>D1X2</td>
<td>2 : 6 : 1 : 0</td>
<td>No</td>
</tr>
<tr>
<td>det1(pUP39a) x det12</td>
<td>D1X12</td>
<td>2 : 5 : 2 : 2</td>
<td>No</td>
</tr>
<tr>
<td>det1(pUP39a) x det13</td>
<td>D1X13</td>
<td>0 : 9 : 0 : 0</td>
<td>No</td>
</tr>
<tr>
<td>det2(pUP39a) x det2</td>
<td>D2X2</td>
<td>9 : 0 : 0 : 0</td>
<td>Yes</td>
</tr>
<tr>
<td>det2(pUP39a) x det12</td>
<td>D2X12</td>
<td>1 : 7 : 1 : 1</td>
<td>No</td>
</tr>
<tr>
<td>det2(pUP39a) x det13</td>
<td>D2X13</td>
<td>8 : 0 : 0 : 0</td>
<td>Yes</td>
</tr>
<tr>
<td>det12(pUP39a) x det12</td>
<td>D12X12</td>
<td>8 : 0 : 0 : 0</td>
<td>Yes</td>
</tr>
<tr>
<td>det12(pUP39a) x det13</td>
<td>D12X13</td>
<td>1 : 5 : 3 : 0</td>
<td>No</td>
</tr>
<tr>
<td>det13(pUP39a) x det13</td>
<td>D13X13</td>
<td>8 : 0 : 0 : 0</td>
<td>Yes</td>
</tr>
<tr>
<td>Strain</td>
<td>β-gal activity in MM + Thi (1.5μM)</td>
<td>Strain</td>
<td>β-gal activity in MM + Thi (1.5μM)</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------------------------------------</td>
<td>--------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>CD1(pUP39a)</td>
<td>0.5 ± 0.3</td>
<td>det12(pUP39a)</td>
<td>659 ± 99</td>
</tr>
<tr>
<td>det1(pUP39a)</td>
<td>781 ± 136</td>
<td>D12X12(pUP39a)</td>
<td>160 ± 49</td>
</tr>
<tr>
<td>D1X1(pUP39a)</td>
<td>145 ± 30</td>
<td>RHD12(pUP39a)</td>
<td>34 ± 9</td>
</tr>
<tr>
<td>RHD1(pUP39a)</td>
<td>28 ± 3</td>
<td>D2X2(pUP39a)</td>
<td>82 ± 14</td>
</tr>
<tr>
<td>det2-2(pUP39a)</td>
<td>635 ± 81</td>
<td>D13X13(pUP39a)</td>
<td>240 ± 51</td>
</tr>
<tr>
<td>D2X2(pUP39a)</td>
<td>82 ± 14</td>
<td>RHD13(pUP39a)</td>
<td>55 ± 16</td>
</tr>
<tr>
<td>RHD2(pUP39a)</td>
<td>0.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.11 *THI4-lacZ* expression in haploid *det* mutants 1, 2-2, 12 and 2-13 compared to their *det/det* homozygous diploid and *DET/det* heterozygous diploid derivatives. All β-galactosidase assays were carried out after growth in medium containing thiamine (1.5μM) and compared to the wild-type strain CD1(pUP39a) as a control.

Figure 4.2 *THI4-lacZ* expression in haploid *det* mutants 1, 2-2, 12 and 2-13 compared to their *det/det* homozygous diploid and *DET/det* heterozygous diploid derivatives. Graphical representation of the β-galactosidase activity data from Table 4.11.
mutants displayed derepressed β-galactosidase activity levels part way between those of the homozygous det/det mutant diploid and the homozygous DET/DET wild-type control diploid. This implied that both the wild-type DET and the mutant det alleles were contributing to give the activity values detected in the heterozygous diploid.

4.8 Discussion

A successful strategy was developed for the isolation of UV induced THI4-lacZ derepressed mutants, based on their appearance as blue coloured colonies on minimal medium containing X-gal and thiamine (1.5 μM). Mutants were isolated both in the haploid strain W303a(pUP39a) and in the diploid strain CD1(pUP39a). As expected the frequency of det mutants detected (as defined by a strain displaying greater than two units of β-galactosidase activity in the presence of thiamine), was lower in the diploid screen than the haploid screen; in the order of 1 in 10,000 compared to 1 in 2,000 colonies screened. This five fold difference reflects the fact that recessive mutations are not detected in a diploid screen.

Thirteen det mutants were isolated in total, none of which were due to pUP39a plasmid-based mutations. This observation is in agreement with preliminary deletion analysis of the THI4 promoter, in which no potential negative acting regulatory sites were found (K. Byrne, pers. comm). It would have been in these negative sites, if they existed, where plasmid-based "operator constitutive" type mutations could result in derepressed expression of the THI4-lacZ reporter gene. This theory could be tested by carrying out in vitro mutagenesis on the pUP39a plasmid itself with hydroxylamine, followed by transformation into yeast and analysis on X-gal medium containing thiamine (Busby et al, 1982). A lack of any mutant blue colonies would imply that the production of derepressed plasmid-based mutations is not possible, and that cis-acting negative regulatory sites within the THI4 promoter are unlikely to exist.

Sporulation and meiotic segregation analysis of the thirteen mutant strains indicated that in eight cases the det mutant phenotype was the result of a mutation in a single gene, and in one case, det11, the result of mutations in more than one gene. The remaining four det mutant strains 3, 4, 7 and 10, all produced diploids that were unable to sporulate after mating with either of the wild-type strains KBY4 or DBY746. These det strains might therefore have also gained a sporulation defect (spo) during the UV mutagenesis. The eight single gene det mutants could be subdivided into two types according to their relative β-galactosidase activity levels in haploids grown in the presence of thiamine (1.5 μM). Type 1 det mutants (5, 6, 8 and 9) displayed β-galactosidase values less than ten units, and type 2 det mutants (1, 2-2, 12 and 2-13) displayed β-galactosidase values greater than 600 units in the presence of thiamine. It is these type 2 mutants that are of particular interest, as they show
derepressed THI4-lacZ expression levels in the presence of thiamine similar to levels recorded from wild-type cells grown in the absence of thiamine.

To classify further the det mutants and determine the number of mutant genes they represented, complementation analysis was carried out. This analysis was complicated by three of the mutations being partially dominant, and the finding that type 1 mutants showed no detectable mutant phenotype in a homozygous diploid state. Homozygous diploids of type 2 det mutants also showed a noticeable decrease in β-galactosidase activity in the presence of thiamine, as did the wild-type control diploid strain grown under non-repressing conditions. This phenomenon could be due to a general difference in metabolism between haploid and diploid cells, the protocol used to assay β-galactosidase activity, or a combination of the two. It is unlikely to be caused by a ploidy effect on THI4 transcriptional control. This is because the THI4 promoter contains no cis-acting sequences known to be involved in regulation in response to the cells ploidy, for example those of the SWI genes required for positive regulation of HO transcription in MATa and MATα cells (Klar, 1987). Recently Schenk et al., (1996) have shown that the cell permeabilisation method used here for assaying β-galactosidase activity gives different ranges of activity between strains when the same reporter gene and growth conditions are used. These differences may be caused by slight changes in the structure and composition of cell walls between strains, which would in turn affect the relative degree of cell permeabilisation. This method is therefore potentially not sensitive enough to detect a det mutant phenotype in type 1 homozygous diploids, after a decrease in activity caused by the change of strain genetic background.

To complete the complementation analysis of the det mutants 5, 6, 8 and 9, the method used for the type 2 mutants could be employed. This would entail sporulating the heterozygous diploids shown in Table 4.9, and determining their det segregation pattern compared to the homozygous diploids. A 4 : 0 pattern would indicate that the two det mutations in that diploid were allelic. Complementation analysis of the type 2 mutants showed that the four det mutants corresponded to mutations within three different genes, that can be represented as (i) det1, (ii) det2-2 and det2-13, and (iii) det12. How the recessive and partially dominant mutations det2-2 and det2-13 could be allelic, and the possible sites of mutation for all four type 2 mutants will be discussed fully in the general discussion chapter 8. What is clear though is that det mutants 1, 2-2, 2-13 and 12, are unlikely to contain mutations within THI80, the only gene known to date that results in a derepressed expression of thiamine genes phenotype when mutant (Nishimura et al, 1991). THI80 encodes the biosynthetic enzyme thiamine pyrophosphokinase, and its derepressed mutant phenotype is caused by an inability of the cell to produce sufficient TPP concentrations to repress thiamine gene expression. These mutations would be recessive in origin, therefore the only det mutant that could be considered as a
candidiate thi80 mutant, would be det2-2. However this cannot be the case as det2-2 is allelic to the partially dominant mutant det2-13.
CHAPTER FIVE
PHENOTYPIC ANALYSIS OF DEREPRESSED THI4-lacZ MUTANTS

5.1 Introduction

Chapter four described the isolation of a number of mutant strains that displayed derepressed expression of the plasmid-based reporter gene THI4-lacZ. This chapter describes further analysis carried out on the four type 2 det mutants (1, 2-2, 12 and 2-13), to characterize them in greater detail at the phenotypic level. Initially this analysis continued to focus upon THI4-lacZ expression within the det mutants, with the questions asked being (i) what lacZ levels would be recorded in the det mutants grown under normal derepressed conditions, i.e. in the absence of thiamine, and (ii) what effects did further increases in exogenous thiamine have. Also by carrying out Northern blot analysis on these mutant strains I could determine whether the chromosomal THI4 locus also displayed derepressed expression.

Studies were also carried out on det mutants 1, 2-2, 12 and 2-13 to determine whether the expression of other known TPP-controlled genes, and the TPP-controlled uptake of thiamine were affected. As with THI4, regulation of other genes could be studied by THI-lacZ reporter gene assays and by Northern blot analysis, under defined conditions of thiamine.

5.2 Analysis of THI4 expression in the type 2 det mutants

5.2.1 THI4-lacZ expression in haploid det mutants 1, 2-2, 12 and 2-13(pUP39a)

Two important questions regarding the expression of THI4-lacZ within the det mutants 1, 2-2, 12 and 2-13, needed to be answered. These were (i) what is the level of THI4-lacZ expression within det mutants grown under conditions of thiamine which would normally cause derepressed expression, and (ii) does the THI4 promoter experience repression at very high thiamine concentrations in the det mutants. β-galactosidase assays were therefore carried out on det1, det2-2, det12 and det2-13, all containing pUP39a, in medium supplemented with 0, 1.5μM and 10μM thiamine (Table 5.1 and figure 5.1).

All four mutant strains grew in the absence of thiamine indicating that they were not thiamine auxotrophs. Furthermore the mean β-galactosidase activity levels recorded under such growth conditions were greater than those of a derepressed wild-type strain, W303a(pUP39a). This implied that either THI4-lacZ expression in W303a(pUP39a) was subject to a degree of regulation in the absence of thiamine that was reduced or overridden in the mutant strains, or that the mutant strains possessed an enhanced level of activation.

When thiamine was present in the growth medium, cultures of the det mutants displayed decreased β-galactosidase activity in response to increases in
Strain | β-galactosidase activity in
| MM - Thiamine | MM + Thi (1.5μM) | MM + Thi (10μM)
---|---|---|---
W303a(pUP39a) | 1060 ± 138 | 0.5 ± 0.2 | 0.5 ± 0.2
\(det1\)(pUP39a) | 1406 ± 379 | 781 ± 136 | 240 ± 46
\(det2-2\)(pUP39a) | 1489 ± 425 | 635 ± 81 | 107 ± 32
\(det12\)(pUP39a) | 1150 ± 274 | 659 ± 99 | 394 ± 99
\(det2-13\)(pUP39a) | 1554 ± 389 | 1327 ± 228 | 1111 ± 295

Table 5.1 THI4-lacZ expression in \(det\) mutants 1, 2-2, 12 and 2-13(pUP39a)

β-galactosidase activity of the \(det\) mutant strains 1, 2-2, 12 and 2-13(pUP39a) and the \(DET\) strain W303a, after growth in medium supplemented with 0, 1.5μM and 10μM thiamine. All assays were carried out at least in triplicate on duplicate samples.

Figure 5.1 THI4-lacZ expression in \(det\) mutants 1, 2-2, 12 and 2-13(pUP39a)

Graphical representation of the β-galactosidase activity data from Table 5.1
concentration from 0 to 1.5μM, and from 1.5μM to 10μM. Therefore despite showing a clear derepressed \( THI4\)-\( lacZ \) expression phenotype at the higher thiamine concentration, all four mutants still showed some level of regulation by the added thiamine. The mutant \( det2-13(pUP39a) \) was most noticeable in that it showed the smallest decreases in \( \beta \)-galactosidase activity with increasing thiamine concentration. Even in the presence of 10μM thiamine, \( THI4\)-\( lacZ \) expression in this mutant was greater than in the induced wild-type strain.

5.2.2 \( THI4\)-\( lacZ \) expression in \( det/DET \) heterozygous diploids RHD1, RHD2, RHD12 and RHD13(pUP39a)

As all four haploid mutants showed decreased \( THI4\)-\( lacZ \) expression with increasing exogenous thiamine concentrations, the four respective \( det/DET \) heterozygous diploid strains were also assayed for \( \beta \)-galactosidase activity in medium supplemented with 0, 1.5μM and 10μM thiamine. This experiment tested whether the partial dominant phenotype of \( det \) mutants 1, 2-2, 12 and 2-13 was still exhibited at a higher thiamine concentration. The results of these assays and those on the wild-type control CD1(pUP39a) are shown in Table 5.2 and figure 5.2.

As shown in chapter four, all diploids gave \( \beta \)-galactosidase activity values less than their haploid counterparts in the absence of thiamine. Of greater interest was the observation that RHD1, RHD12 and RHD13(pUP39a) all showed lower \( \beta \)-galactosidase activities in medium containing 10μM thiamine than in medium containing 1.5μM thiamine; however they still exhibited a clear derepressed \( THI4\)-\( lacZ \) expression phenotype. This proved that \( det1, \ det12 \) and \( det2-13 \) all possessed a partial dominant mutant phenotype.

5.2.3 Chromosomal \( THI4 \) expression in \( det \) mutants 1, 2-2, 12 and 2-13

Thus far all analysis of \( THI4 \) expression in the \( det \) mutants had been carried out by assaying for expression of the pUP39a plasmid-based \( THI4\)-\( lacZ \) reporter gene. To check that the chromosomal \( THI4 \) locus was also derepressed in these mutants, Northern blot analysis was carried out on \( det1, \ det2-2, \ det12 \) and \( det2-13 \) grown in the presence of thiamine. As a control the \( DET^+ \) strain W303a was assayed after growth in the presence and absence of thiamine. Duplicate filters carrying total cellular RNAs were hybridised separately with (i) DNA from the \( ACT1 \) gene, and (ii) the \( THI4 \) cDNA (figure 5.3).

As expected, W303a displayed \( THI4 \) expression in the absence of thiamine but no \( THI4 \) mRNA was detectable in cells grown with thiamine. In contrast, the four mutant strains all displayed \( THI4 \) expression in the presence of thiamine. In agreement with the \( \beta \)-galactosidase activity data (Table 5.1), the derepressed levels of \( THI4 \) mRNA detected in \( det \) mutants were comparable to W303a grown in the
Strain P-galactosidase activity in

<table>
<thead>
<tr>
<th>Strain</th>
<th>MM - Thiamine</th>
<th>MM + Thi (1.5μM)</th>
<th>MM + Thi (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1(pUP39a)</td>
<td>804 ± 107</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>RHD1(pUP39a)</td>
<td>571 ± 107</td>
<td>28 ± 3</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>RHD2(pUP39a)</td>
<td>619 ± 146</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>RHD12(pUP39a)</td>
<td>530 ± 138</td>
<td>34 ± 9</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>RHD13(pUP39a)</td>
<td>410 ± 89</td>
<td>55 ± 16</td>
<td>38 ± 12</td>
</tr>
</tbody>
</table>

Table 5.2 THI4-lacZ expression in RHD1, RHD2, RHD12 and RHD13(pUP39a)

β-galactosidase activity of the det/DET heterozygous diploid strains RHD1, RHD2, RHD12 and RHD 13(pUP39a) and the DET/DET strain CD1(pUP39a) in medium supplemented with 0, 1.5μM and 10μM thiamine. All assays were carried out at least in triplicate on duplicate samples.

Figure 5.2 THI4-lacZ expression in RHD1, RHD2, RHD12 and RHD13(pUP39a)

Graphical representation of the β-galactosidase activity data from Table 5.2.
Figure 5.3 Northern blot analysis of *THI4* expression in *det* mutants 1, 2-2, 12 and 2-13

Total cellular RNAs isolated from W303a grown in the presence (1.5μM) and absence of thiamine, and from *det* mutants 1, 2-2, 12 and 2-13 grown in the presence of thiamine, were hybridised with probes specific for *ACT1* and *THI4*

Lane 1: W303a grown in MM - Thiamine
Lane 2: W303a grown in MM + Thiamine (1.5μM)
Lane 3: *det1* grown in MM + Thiamine (1.5μM)
Lane 4: *det2-2* grown in MM + Thiamine (1.5μM)
Lane 5: *det12* grown in MM + Thiamine (1.5μM)
Lane 6: *det2-13* grown in MM + Thiamine (1.5μM)
absence of thiamine. Mutants det1, det2-2, det12 and det2-13 were therefore derepressed for the chromosomal THI4 locus as well as the THI4-lacZ reporter gene.

5.3 Expression of other TPP-regulated genes in det mutants 1, 2-2, 12 and 2-13

The question arose as to whether the mutations in det1, det2-2, det12 and det2-13 were specific to THI4, or whether they were pleiotropic for all TPP-regulated genes. Analysis was therefore carried out into the expression of THI5, THI6, THI12, THI80 and PHO3 within the mutant strains. This analysis involved the use of Northern blot experiments, and with the exception of PHO3, reporter gene studies to determine whether thiamine gene expression was wild-type or derepressed.

5.3.1 Construction of reporter genes

As described in section 3.2, THI5-lacZ and THI12-lacZ reporter genes were produced using a PCR strategy involving the 'add-on' of restriction sites, to enable cloning of thiamine gene fragments into pUP34. Using this same strategy, but with primers specific to THI6 and THI80, the THI6-lacZ and THI80-lacZ reporter gene plasmids pLK2 and pLK3 were also constructed in this laboratory (Kew, 1996). Restriction maps of these plasmids are shown in figure 5.4, and details of their production, compared to pRB3 and pRB4, are shown in Table 5.3.

5.3.2 Use of lacZ reporter constructs to monitor THI gene regulation in det mutants 1, 2-2, 12 and 2-13

The four plasmid free det mutant strains and W303a were each transformed with plasmids pRB3 (THI5-lacZ), pRB4 (THI12-lacZ), pLK2 (THI6-lacZ) and pLK3 (THI80-lacZ) and transformants selected on medium lacking uracil. β-galactosidase assays were then carried out on liquid cultures of the transformants grown in the presence (1.5μM) and absence of thiamine; results of all these assays are shown in Table 5.4 and figures 5.5 and 5.6.

In agreement with THI6 Northern blot analysis (Nosaka et al, 1994), W303a(pLK2) displayed high levels of β-galactosidase activity in the absence of thiamine (150 units), compared to 1 unit in the presence of thiamine. This indicated that the THI6 promoter was functional and that all the sequences necessary for regulation in response to added thiamine were present. In comparison the THI80-lacZ construct showed lower levels of β-galactosidase in the absence of thiamine (30 units), and less than 1 unit in the presence of thiamine. This was somewhat surprising since Northern blot experiments carried out by Nosaka et al (1993) had shown that THI80 was expressed in the absence of thiamine, but also at a lower basal level in the presence of 1μM.
Figure 5.4 Plasmid maps of pLK2 and pLK3
<table>
<thead>
<tr>
<th>Reporter gene construct</th>
<th>PCR template</th>
<th>Region of THI gene cloned into pUP34</th>
<th>Junction sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>THI5-lacZ (pRB3)</td>
<td>pRH11</td>
<td>-667 to +87 Asp Val Thr Gly Asp Pro Val Val Leu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAA GGT TAC GGG GAT CCC GTC GTT TTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>THI5(12) -&gt; BamHI lacZ -&gt;</td>
<td></td>
</tr>
<tr>
<td>THI12-lacZ (pRB4)</td>
<td>pSK+1.1</td>
<td>-667 to +87 Asp Val Thr Gly Asp Pro Val Val Leu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAA GGT TAC GGG GAT CCC GTC GTT TTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>THI5(12) -&gt; BamHI lacZ -&gt;</td>
<td></td>
</tr>
<tr>
<td>THI6-lacZ (pLK2)</td>
<td>pKB16</td>
<td>-629 to +33 Asp Tyr Ser Leu Asp Pro Val Val Leu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAT TAC TCA TTG GAT CCC GTC GTT TTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>THI6 -&gt; BamHI lacZ -&gt;</td>
<td></td>
</tr>
<tr>
<td>THI80-lacZ (pLK3)</td>
<td>pAN8</td>
<td>-756 to +21 Cys Ile Glu Lys Asp Pro Val Val Leu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGT ATT GAA AAG GAT CCC GTC GTT TTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>THI80 -&gt; BamHI lacZ -&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 Table summarising the four PCR cloned THI-lacZ reporter gene constructs. Details shown are the plasmid used as the PCR template, the region of the thiamine gene cloned into pUP34 and the sequence across the translational fusion junction. The numbers shown above amino acids indicate the codon position they occupy in their respective genes. Amino acids that are not numbered represent new codons formed by the fusion. The homologous genes THI5 and THI12 are listed together as the same primers were used to produce both constructs. Constructs pLK2 and pLK3 were made by L.Kew (Kew, 1996).
### Table 5.4 THI-lacZ expression in the DET strain W303a and the det mutant strains 1, 2-2, 12 and 2-13

<table>
<thead>
<tr>
<th>Strain</th>
<th>pRB3 (THI5-lacZ)</th>
<th>pRB4 (THI12-lacZ)</th>
<th>pLK2 (THI6-lacZ)</th>
<th>pLK3 (THI80-lacZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Thi</td>
<td>+ Thi</td>
<td>- Thi</td>
<td>+ Thi</td>
</tr>
<tr>
<td>W303a</td>
<td>428 ± 112</td>
<td>0.6 ± 0.2</td>
<td>452 ± 113</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>det1</td>
<td>287 ± 67</td>
<td>51 ± 17</td>
<td>260 ± 49</td>
<td>51 ± 12</td>
</tr>
<tr>
<td>det2-2</td>
<td>452 ± 110</td>
<td>5.0 ± 1.0</td>
<td>437 ± 125</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>det12</td>
<td>483 ± 120</td>
<td>35 ± 7</td>
<td>367 ± 43</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>det2-13</td>
<td>422 ± 79</td>
<td>10 ± 2</td>
<td>439 ± 185</td>
<td>19 ± 6</td>
</tr>
</tbody>
</table>

β-galactosidase activity of W303a, det1, det2-2, det12 and det2-13, each transformed with plasmids pRB3, pRB4, pLK2 and pLK3 and grown in the presence (1.5μM) and absence of thiamine. All assays were carried out at least in triplicate upon duplicate samples.
Figure 5.5  THI5-lacZ and THI12-lacZ expression in det mutants 1, 2-2, 12 and 2-13
Graphical representation of (A) the THI5-lacZ and (B) the THI12-lacZ 
β-galactosidase activity data from Table 5.4
Figure 5.6 THI6-lacZ and THI80-lacZ expression in det mutants 1, 2-2, 12 and 2-13

Graphical representation of (A) the THI6-lacZ and (B) the THI80-lacZ β-galactosidase activity data from Table 5.4
thiamine. Possible reasons for the apparent lack of basal activity in my experiment will be discussed at the end of this chapter. However for the purpose of this experiment I was more concerned with how THI80-lacZ expression was affected by the det mutations.

All four mutant strains displayed derepressed expression of the four THI-lacZ reporter constructs in cultures grown with added thiamine (1.5μM), indicating that the mutations were pleiotropic for TPP-regulated genes. Interestingly though, the degree of derepression from a given reporter construct in any of the mutant strains was not as great as that seen with the original THI4-lacZ reporter gene. Each THI-lacZ construct also showed its own hierarchical pattern for the level of derepressed expression within the four mutants; det1 and det12 showed the highest derepressed β-galactosidase activities with pRB3 and pRB4, and det2-2 and det2-13 showed the highest derepressed β-galactosidase activities with pLK2.

5.3.3 Northern blot analysis of THI gene expression in det mutants1,2-2,12 and 2-13

Northern blot analysis was carried out to check that the chromosomal THI5, THI12, THI6 and THI80 loci, in addition to their respective reporter genes, were derepressed in the det mutants. As in section 5.2.3, mRNA levels in cultures of det1, det2-2, det12 and det2-13 grown in the presence of 1.5μM thiamine, were compared to those from W303a grown in the presence and absence of thiamine. Filters carrying total cellular RNAs were hybridised separately with (i) DNA from the ACT1 gene, (ii) the THI5 cDNA, (iii) the THI6 gene, (iv) the THI80 gene and (v) the PHO3 gene. The results of these hybridisations are shown in figure 5.7.

As expected the TPP-regulated genes THI5, THI6, THI80 and PHO3 were all highly expressed in the wild-type W303a strain grown in the absence of thiamine, whereas there was little or no expression in cells grown with thiamine. A low level of expression in the presence of thiamine was most noticeable for THI80 and THI6, and was consistent with the Northern blot analysis carried out by Nosaka et al (1993 and 1994). The various genes examined also showed differences in their relative levels of transcriptional expression in W303a grown in the absence of thiamine, as determined by the average length of autoradiograph exposure time required prior to development of the filters. THI4 (fig 5.3) and THI5 were the most highly expressed thiamine genes, requiring less than 12 hours exposure time at minus 80°C prior to development. PHO3 was expressed at similar levels to ACT1, in that filters were standardly exposed for two to three days prior to development. The two remaining genes, THI6 and THI80, were expressed at low levels even in the absence of thiamine, as filters had to be left for up to a week at minus 80°C prior to development.
Figure 5.7 Northern blot analysis of THI gene expression in det mutant strains det1, 2-2, 12 and 2-13

Total cellular RNAs isolated from W303a grown in the presence (1.5μM) and absence of thiamine, and from det mutants grown in the presence of thiamine, were hybridised with probes specific for ACT1, THI5, THI6, THI80 and PHO3

Lane 1 : W303a grown in MM - Thiamine
Lane 2 : W303a grown in MM + Thiamine (1.5μM)
Lane 3 : det1 grown in MM + Thiamine (1.5μM)
Lane 4 : det2-2 grown in MM + Thiamine (1.5μM)
Lane 5 : det12 grown in MM + Thiamine (1.5μM)
Lane 6 : det2-13 grown in MM + Thiamine (1.5μM)
In agreement with the reporter gene data (Table 5.4), all four det mutant strains showed derepressed transcriptional expression of THI5, THI6, and THI80. They also showed derepressed expression of PHO3, therefore supporting the conclusion that the mutations det1, det2-2, det12 and det2-13, were pleiotropic for all TPP-regulated genes.

5.3.4 Expression of a non-thiamine gene in det mutants 1, 2-2, 12 and 2-13

Available within the laboratory was the plasmid pUP40, which contained the CYC1 promoter and ATG start codon fused in frame with the lacZ gene of pUP34 (U. Praekelt pers.comm.). Expression of CYC1, which encodes iso-1-cytochrome c, is regulated by the concentration of heme in the growth medium and not thiamine (Guarente and Mason, 1983; Guarente et al, 1984). This construct was therefore used as a control to check whether the expression of a non-thiamine gene was affected in the det mutants.

The four det mutants were transformed with pUP40 and β-galactosidase assays carried out on cultures grown in the presence of 1.5μM thiamine, compared to W303a(pUP40) grown in the presence and absence of thiamine. Expression of CYC1-lacZ was not influenced by thiamine concentration, as W303a(pUP40) gave approximately 45 units of β-galactosidase activity with and without thiamine (Table 5.5 and figure 5.8). The four mutant strains also gave β-galactosidase activity values of around 45 units in the presence of thiamine, indicating that the det mutations had no effect on CYC1 expression.

5.4 Thiamine transport in det mutants 1, 2-2, 12 and 2-13

It is possible that the det strains 1, 2-2, 12 and 2-13 were the result of mutations not in thiamine regulatory genes, but instead in genes required for thiamine transport. Therefore cells grown in medium containing thiamine would be unable to take up the vitamin, and intracellular TPP levels would not become repressing to thiamine gene expression. Alternatively the det mutants may contain mutations within the thiamine pyrophosphokinase gene THI80. Here thiamine transport would still occur, however conversion into the negative effector TPP would be reduced such that again thiamine genes were not repressed. I therefore decided to study thiamine uptake in the det mutants, in order to determine whether either of these possibilities was occurring. Unfortunately the thiochrome method employed here to determine thiamine concentrations does not distinguish between thiamine, thiamine monophosphate or TPP levels within the cell, therefore it would not be possible to check whether the mutants were allelic to THI80 (Tommasino and Maundrel, 1991). Such analysis would require HPLC to determine individual concentrations of thiamine and its phosphate esters, and this was not available to me.
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM - Thiamine</th>
<th>MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pUP40)</td>
<td>45 ± 4</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>det1(pUP40)</td>
<td>nt</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>det2-2(pUP40)</td>
<td>nt</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>det12(pUP40)</td>
<td>nt</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>det2-13(pUP40)</td>
<td>nt</td>
<td>42 ± 11</td>
</tr>
</tbody>
</table>

Table 5.5 CYC1-lacZ expression in det mutants 1, 2-2, 12 and 2-13(pUP40)

β-galactosidase activity of det mutants 1, 2-2, 12 and 2-13(pUP40) grown in the presence of thiamine (1.5μM), compared to W303a(pUP40) grown in the presence and absence of thiamine. All assays were carried out at least in triplicate on duplicate samples. nt indicates not tested.

Figure 5.8 CYC1-lacZ expression in det mutants 1, 2-2, 12 and 2-13(pUP40)

Graphical representation of the β-galactosidase activity data from Table 5.5
In addition to studying thiamine uptake, it was also of interest to measure the basal intracellular levels of thiamine within the det mutants. This was because the strains det1, det2-2, det12 and det2-13 were derepressed for all thiamine biosynthetic genes, therefore the question arose as to whether intracellular thiamine levels were greater as a result.

5.4.1 Basal intracellular thiamine concentrations in det mutants 1, 2-2, 12 and 2-13

To check that the det mutants contained normal intracellular thiamine levels, their basal intracellular thiamine concentration was measured compared to the DET+ strain W303a. This analysis required prolonged growth of each strain in medium completely devoid of thiamine prior to the assay of thiamine concentration. Therefore the det strains 1, 2-2, 12 and 2-13, and the control strain W303a were grown overnight in medium lacking thiamine, back diluted to a concentration of 1x10^5 cells/ml in 150ml fresh medium lacking thiamine, and regrown to a cell density of 2x10^7 cells/ml. At this point cultures were centrifuged and the basal intracellular thiamine concentration determined by measuring the concentration of thiamine extracted from the cell pellets. The extracellular thiamine concentrations were also determined by measuring the thiamine content of the culture supernatants (see Table 5.6).

In agreement with previous intracellular thiamine assays, the wild-type strain W303a displayed a basal concentration of 8 pmol/10^7 cells (Praekelt et al., 1994). Of the mutant strains, det1, det2-2 and det2-13 showed intracellular thiamine concentrations close to that of W303a, implying that the rate of thiamine monophosphate production in these strains was wild-type. In contrast, the mutant det12 showed approximately half the intracellular level recorded for W303a. This suggested that the phenotype of det12 might be the result of a reduced ability to synthesise TPP.

None of the mutant strains tested excreted thiamine into the extracellular environment. Thus the det mutations did not generate thiamine-secreting yeast strains, which would be of considerable interest to the food industry (Haj-Ahmad et al., 1992).

5.4.2 Thiamine uptake in det mutants 1, 2-2, 12 and 2-13

To determine whether the det mutants contained a functional thiamine transport system, their ability to take up thiamine was compared to the DET+ strain W303a. Cultures (10ml) of strains W303a, det1, det2-2, det12 and det2-13 were grown overnight in medium lacking thiamine, back diluted to 1x10^5 cells/ml in 150ml fresh medium lacking thiamine, and regrown to 2x10^7 cells/ml. At this point (time 0), two 10ml samples were taken from each culture, and thiamine added to the remaining medium to a final concentration of 1.5μM.
Table 5.6 Intracellular and extracellular concentrations of thiamine in W303a and det mutants 1, 2-2, 12 and 2-13

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular (pmoles/10^7 cells)</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a</td>
<td>8.3 ± 2.4</td>
<td>0.0</td>
</tr>
<tr>
<td>det1</td>
<td>6.8 ± 2.4</td>
<td>0.0</td>
</tr>
<tr>
<td>det2-2</td>
<td>10.9 ± 2.4</td>
<td>0.0</td>
</tr>
<tr>
<td>det12</td>
<td>4.5 ± 1.7</td>
<td>0.0</td>
</tr>
<tr>
<td>det2-13</td>
<td>6.3 ± 1.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Strains W303a, det1, det2-2, det12 and det2-13 were grown overnight in medium lacking thiamine, back diluted to a concentration of 1x10^5 cells/ml in 150ml fresh medium lacking thiamine, and regrown to a cell density of 2x10^7 cells/ml. Cultures were then pelleted and the intracellular and extracellular thiamine concentration determined for each strain. All assays were carried out in triplicate on duplicate samples.
Further duplicate 10ml samples were taken at times 0.5, 1, 2, 3, 4 and 10.5 hours after thiamine addition, and assays carried out to determine the intracellular and extracellular thiamine concentrations. The intracellular thiamine data from this uptake experiment are shown in Table 5.7 and figure 5.9.

Data from the extracellular thiamine assays has not been shown because at all time points tested, no thiamine was detected in the extracellular medium of any of the five strains. This indicated that each det mutant had no obvious defect in thiamine accumulation, as all exogenous thiamine was exhausted from the culture medium within 30 minutes of its addition. Again this was in agreement with previous work (Praekelt et al., 1994), which found that 1μM thiamine was completely sequestered from the extracellular medium within 30 minutes in a wild-type strain. Following uptake, W303a, det1, det2-2 and det2-13 showed a gradual decrease in intracellular thiamine concentration with time, as the vitamin became diluted by an increase in biomass. In comparison the intracellular thiamine concentration of det12 decreased at a much greater rate, such that 10 hours after thiamine uptake the thiamine concentration had returned to this strains basal level. The fact that det12 did not excrete thiamine back into the medium, implied that the thiamine was being either degraded or localized within the cell in such a way, as to be unavailable to detection by this method.

5.4.3 Northern blot analysis of THI4 expression following thiamine uptake

The observations that strain det12 possessed a reduced basal intracellular thiamine concentration and that it appeared to 'lose' transported thiamine, questioned whether this strain was mutant for a thiamine regulatory gene. To therefore prove that the det mutants displayed derepressed THI4 expression when intracellular thiamine concentrations were high, the following Northern blot experiment was carried out. Duplicate 10 ml cultures of strains W303a, det1, det2-2, det12 and det2-13 were grown to a cell density of 2x10^7 cells/ml in medium lacking thiamine. Thiamine was then added to a final concentration of 1.5μM to one culture of each strain, and both cultures allowed to grow for another hour. Total RNA was then prepared from all ten cultures and Northern blot analysis carried out using ACT1 and THI4 DNA as probes (figure 5.10).

From the data in figure 5.9, it was known that all five strains possessed an intracellular thiamine concentration in excess of 200 pmol/10^7 cells one hour after the addition of thiamine to the growth medium. This concentration was ten times greater than the value previously determined to be the the minimum concentration required for repression of THI4 (Praekelt et al., 1994). Therefore, assuming that the strain was wild-type for THI80 and the thiamine taken up was converted into TPP, no THI4 expression should be detected. This was indeed the
<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular thiamine concentration (pmol/10^7 cells) at time (hr)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>10.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a</td>
<td></td>
<td>8.0</td>
<td>467</td>
<td>363</td>
<td>266</td>
<td>260</td>
<td>258</td>
<td>137</td>
</tr>
<tr>
<td>det1</td>
<td></td>
<td>12.9</td>
<td>494</td>
<td>425</td>
<td>489</td>
<td>450</td>
<td>370</td>
<td>284</td>
</tr>
<tr>
<td>det2-2</td>
<td></td>
<td>11.9</td>
<td>429</td>
<td>289</td>
<td>315</td>
<td>259</td>
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</tr>
<tr>
<td>det12</td>
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<td>3.9</td>
<td>336</td>
<td>234</td>
<td>299</td>
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<td>125</td>
<td>2.1</td>
</tr>
<tr>
<td>det2-13</td>
<td></td>
<td>7.6</td>
<td>332</td>
<td>262</td>
<td>334</td>
<td>270</td>
<td>230</td>
<td>191</td>
</tr>
</tbody>
</table>

Table 5.7 Thiamine uptake in W303a and det mutants 1, 2-2, 12 and 2-13

Strains W303a, det1, det2-2, det12 and det2-13 were grown overnight in medium lacking thiamine, back diluted to 1x10^5 cells/ml in 150ml fresh medium lacking thiamine, and regrown to 2x10^7 cells/ml. At this point (time 0) thiamine was added to a final concentration of 1.5μM. Cell samples were taken at 0, 0.5, 1, 2, 3, 4 and 10.5 hours and assayed for intracellular thiamine concentration. The data shown are the mean of duplicate samples and the experimental error was less than 20%.
Figure 5.9 Thiamine uptake in \textit{det} mutants 1, 2-2, 12 and 2-13
All strains were grown to $2 \times 10^7$ cells/ml in medium lacking thiamine. At time $t=0$ thiamine was added to a final concentration of 1.5$\mu$M. Cell samples were taken at 0, 0.5, 1, 2, 3, 4 and 10.5 hours and assayed for intracellular thiamine concentration. 
\textbf{Graph A} shows thiamine uptake in W303a, \textit{det1} and \textit{det12} and \textbf{graph B} shows thiamine uptake in W303a, \textit{det2-2} and \textit{det2-13}
Figure 5.10 Northern blot analysis of \textit{THI4} expression in strains W303a, \textit{det1,2-2,12} and 2-13 one hour after the addition of thiamine
Lane 1: W303a grown in MM - Thiamine
Lane 2: \textit{detl} grown in MM - Thiamine
Lane 3: \textit{det2-2} grown in MM - Thiamine
Lane 4: \textit{det12} grown in MM - Thiamine
Lane 5: \textit{det2-13} grown in MM - Thiamine
Lane 6: W303a one hour after the addition of thiamine to 1.5\textmu M
Lane 7: \textit{detl} one hour after the addition of thiamine to 1.5\textmu M
Lane 8: \textit{det2-2} one hour after the addition of thiamine to 1.5\textmu M
Lane 9: \textit{det12} one hour after the addition of thiamine to 1.5\textmu M
Lane 10: \textit{det2-13} one hour after the addition of thiamine to 1.5\textmu M
case for W303a, as THI4 mRNA was detected after growth in the absence of thiamine, but this expression was completely repressed by one hour of growth after the addition of thiamine. Although all four mutant strains showed a decrease in THI4 mRNA levels after thiamine addition, they were still clearly displaying derepressed THI4 expression. Thus the det mutants were indeed showing derepressed THI4 expression when TPP levels within the cell would be repressing. Curiously of all the mutants tested, det12 showed the highest level of THI4 expression in the presence of added thiamine.

5.5 Discussion

Using a combination of Northern blot analysis and lacZ reporter gene studies, it has been shown that the det mutants 1, 2-2, 12 and 2-13 display derepressed expression of not only THI4, but also THI5, THI6, THI12, THI80 and PHO3. The mutations in the four mutant strains are therefore not specific to the regulation of THI4, but are instead pleiotropic for all TPP-regulated genes examined. Results with a CYC1-lacZ reporter gene and also the ACT1 loading control during Northern blot analysis, showed that the det mutations are thiamine gene specific. However with the recent discovery of an involvement of PDC2 in the activation of thiamine genes, it is possible that the mutant strains might display derepressed expression of other unrelated genes as well. These could include PDC1 and PDC5, two of the structural genes of pyruvate decarboxylase that are dependent upon PDC2 for expression (Hohmann, 1993). It will therefore be of interest to determine how PDC1 and PDC5 are expressed in the det mutants, compared to a DET+ control strain. The results of this analysis will either support or disprove the hypothesis that det mutations 1, 2-2, 12 and 2-13 affect only thiamine genes, and also provide further insight into the possible overlapping regulatory systems for PDC and THI genes.

When comparing derepressed expression levels from a given thiamine reporter gene in a det mutant, with the corresponding value gained for W303a in the absence of thiamine, it was clear that the most pronounced mutant phenotype occurred with THI4-lacZ. Thus although the mutants were pleiotropic, showing derepression of several TPP-regulated genes, the greatest level of derepression was for THI4-lacZ, the very reporter gene used in the initial selection procedure. A similar observation was gained after mutagenesis screens looking for mutants showing derepressed expression of THI5-lacZ and THI6-lacZ (Kew, 1996). Here mutants also showed derepressed expression of several TPP-regulated genes, but in the majority of cases the extent of derepression was not as great as that of the original reporter gene construct used in the selection process. These results suggest that although the same factors are involved in the
regulation of thiamine genes, the exact complex and interactions they form at a
given thiamine gene promoter, appear to be unique to that gene.

Additional analysis of det1, det2-2, det12 and det2-13 found that they are
thiamine prototrophs, and that thiamine uptake is not compromised. Therefore
providing they are not thi80 mutants, the derepressed expression phenotype they
display does occur when TPP levels within the cell are repressing. Measurement
of each det strain's basal intracellular thiamine concentration found that despite
displaying derepressed expression of all thiamine biosynthetic genes tested, they
did not contain increased levels of thiamine. This observation suggests that
S.cerevisiae possesses mechanisms other than transcriptional regulation for
controlling thiamine production. For example thiamine genes may be subject to
a degree of translational control, such that the derepressed mRNA production
does not correlate with an increase in protein production. Alternatively, the
produced protein may be controlled at the level of enzyme activity.

Finally, the production and subsequent analysis of THI6-lacZ and THI80-
lacZ reporter genes, raised the question of whether the 0.5 - 1.0 unit of β-
galactosidase activity they displayed in the presence of thiamine reflected a low
level of expression or complete repression. This is because Northern blot analysis
of THI6 and THI80 clearly showed that these two genes were transcribed to a low
level in medium containing thiamine. Separate studies by Ruby et al, (1983) and
Struhl et al, (1981) found that an expression value of 0.67 units from HIS3-lacZ
under repressing conditions corresponded to 1 - 2 copies of mRNA per cell. Also
in this laboratory β-galactosidase assays on pUP34, the plasmid containing lacZ
but no promoter, always gave values of 0.2 units or less (K. Byrne, pers. comm.).
Therefore it would appear that β-galactosidase values of above 0.2 units do reflect
a level of promoter driven expression, albeit very low.
CHAPTER SIX
STRATEGIES USED TO CLONE DET ALLELES

6.1 Introduction

Eight strains have been isolated that each displayed a det phenotype due to a mutation within a single nuclear gene. In five cases the det mutation was recessive, and in the other three partially dominant. Complementation analysis has shown that these mutations were present within at least three different genes. In order to identify which genes were mutated in these strains, I decided to attempt to clone the wild-type DET alleles.

Three strategies were employed to determine DET alleles, the first being a candidate gene approach. As det strains appeared to be defective in thiamine gene regulation, a candidate gene would be one that had a role in the regulation of thiamine gene expression. The det strains were therefore transformed with plasmids carrying either THI80 or PDC2, and complementation of the derepressed THI4-lacZ expression phenotype assayed.

The second cloning strategy was that of mutant rescue using a genomic DNA library carried on a plasmid vector (Rose and Broach, 1991). The det strains were transformed with the plasmid-based library, and complementation of the mutant phenotype screened for by detection of wild-type white coloured colonies on X-gal medium containing thiamine. This screen was therefore a reversal of the original mutagenesis screen. Clones conferring a white colony colour on the det strain were taken on for further analysis to determine the identity of the DET gene.

The final cloning strategy involved the construction of a plasmid-based genomic library using genomic DNA isolated from a strain carrying a partially dominant det mutation. This library was then transformed into a wild-type DET+ strain and instead of screening for complementation, I looked for conferment of the det mutant phenotype onto the wild-type strain. This was achieved by screening for blue coloured colonies on X-gal medium containing thiamine, as in the original mutagenesis screen. Clones conferring the mutant blue coloured phenotype would be analysed further to identify the DET gene.

6.2 Are det1, det2-2, det12 and det2-13 allelic with THI80 or PDC2?

To determine whether the det mutants 1, 2-2, 12 and 2-13 contained mutations within THI80 or PDC2, derivatives of each strain that carried the THI4-lacZ reporter gene plasmid pUP39a, were transformed separately with plasmids pRB7 (THI80) and pER1 (PDC2). To check that plasmid pRB7, which I had constructed from pAN8 and pRS315 (see figure 6.1) contained a functional THI80 gene, it was transformed into the thi80-1 mutant strain T48-2D(pUP39a). β-
Figure 6.1 Diagram showing the formation and structure of pRB7
Plasmid pAN8 was digested with SpeI and XhoI, and the 3.1Kb fragment containing _THI80_ ligated into similarly digested pRS315, to produce pRB7.
galactosidase assays were then carried out on the det mutants containing either pRB7 or pER1, T48-2D(pUP39a) and T48-2D(pUP39a, pRB7), in the presence of 1.5μM thiamine (results shown in Table 6.1).

From these data it was clear that pRB7 contained a functional THI80 gene, as it complemented the thi80-l mutant phenotype; T48-2D(pUP39a, pRB7) displayed wild-type repressed levels of β-galactosidase activity in the medium containing thiamine. In comparison, pRB7 had no effect on THI14-lacZ expression in the det mutant strains. These strains containing both pUP39a and pRB7, still displayed derepressed β-galactosidase activities greater than 500 units, indicating that det1, det2-2, det12 and det2-13 were not allelic with THI80.

The plasmid pER1 was also unable to complement the derepressed expression phenotype of the det mutant strains, indicating that det1, det2-2, det12 and det2-13 were not allelic with PDC2.

6.3 Cloning wild-type DET alleles using a plasmid-based genomic DNA library

The second method used to clone DET alleles involved the transformation of a det mutant strain carrying pUP39a, with a plasmid-based yeast genomic DNA library. Transformants were screened on X-gal medium containing thiamine for those that showed a reversal of the det phenotype i.e. gave wild-type white coloured colonies. To check that the white colonies had been caused by a restoration of wild-type reporter gene regulation, quantitative β-galactosidase assays were carried out on each clone in liquid medium containing thiamine (1.5μM). If wild-type regulation had indeed been restored, the transforming plasmid in these clones should carry a dominant wild-type allele of the DET gene.

6.3.1 Choice and validation of the genomic library

Available to me were two plasmid-based genomic DNA libraries. The first was based upon the low copy vector YCp50 (Rose et al, 1987), and the second upon the multicopy vector YEpl3 (Nasmyth and Tatchell, 1980). Ideally the library of choice would have been the one based upon YCp50. This was because being a single copy vector, any clones isolated that displayed wild-type THI14-lacZ expression would have been due to the wild-type DET allele complementing the mutation, and not due to an alternative gene suppressing the mutant phenotype. However as this screen relied upon the expression of a reporter gene also present on a URA3 YCp50 based plasmid, transformation and URA+ selection of both the library plasmid and pUP39a was not possible. Therefore the YEpl3 based library which used a LEU2 selectable marker gene was chosen instead. This library was constructed by the ligation of random 5-20kb sized fragments of yeast AB320 genomic DNA, obtained by Sau3A partial digestion, into the unique BamHI site in YEpl3 (Nasmyth and Tatchell, 1980). The fact that the vector used in this
### Table 6.1 THI4-lacZ expression in det strains 1, 2-2, 12 and 2-13(pUP39a) following transformation with pRB7 and pER1

Mutant strains det1, det2-2, det12 and det2-13 that contained pUP39a, were transformed separately with pRB7(THI80) and pER1(PDC2). β-galactosidase assays were then carried out on cultures of these transformant strains, and T48-2D(pUP39a) and T48-2D(pUP39a, pRB7) grown in the presence of thiamine (1.5μM). All assays were carried out at least in duplicate on duplicate samples.

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T48-2D(pUP39a)</td>
<td>74 ± 17</td>
</tr>
<tr>
<td>T48-2D(pUP39a, pRB7)</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>det1(pUP39a, pRB7)</td>
<td>573 ± 195</td>
</tr>
<tr>
<td>det2-2(pUP39a, pRB7)</td>
<td>464 ± 72</td>
</tr>
<tr>
<td>det12(pUP39a, pRB7)</td>
<td>571 ± 133</td>
</tr>
<tr>
<td>det2-13(pUP39a, pRB7)</td>
<td>867 ± 165</td>
</tr>
<tr>
<td>det1(pUP39a, pER1)</td>
<td>768 ± 145</td>
</tr>
<tr>
<td>det2-2(pUP39a, pER1)</td>
<td>908 ± 171</td>
</tr>
<tr>
<td>det12(pUP39a, pER1)</td>
<td>1068 ± 168</td>
</tr>
<tr>
<td>det2-13(pUP39a, pER1)</td>
<td>1562 ± 242</td>
</tr>
</tbody>
</table>
library was multicopy increased the chances of isolating suppressors of the det mutant phenotype, however in this study this was not considered to be too great a drawback. This was because the aim of the project was to examine thiamine regulation, and the isolation of suppressor genes could therefore open additional areas of study.

Prior to using the YEp13 library to clone DET alleles, it was first tested to determine the frequency of complementation of a single copy recessive auxotrophic mutation, his3. This result established the frequency at which DET+ white colonies should be detected when the library was transformed into det strains, and also tested that the library was functional. The strain W303a which carries his3 and leu2 mutations was therefore transformed with the YEp13 library using the high efficiency method of Schiestl and Geitz (1989), and transformants selected on medium lacking leucine and histidine. As a positive control to calculate the transformation frequency, the transformation mixture was also plated onto medium lacking leucine but containing histidine. As a negative control, untransformed W303a was also plated onto medium lacking leucine and histidine.

It was found that transformation of W303a with the library DNA yielded HIS+ transformants at a frequency of $6 \frac{\text{HIS}^{+}}{19,600 \text{LEU}^{+}}$, i.e. 1 in 3,000 transformants. Given the size of genomic DNA inserts within this library, this frequency of complementation would be expected for a functional library that covered the entire yeast genome.

6.3.3 Choice of det strain for this cloning strategy

Two main factors affected the choice of det strain to use in this cloning strategy. The first was that the strain should contain a recessive det mutation, so that complementation by the YEp13 based library would be possible. However when carrying out β-galactosidase assays on a det/DET heterozygous diploid partially dominant mutant strain compared to its haploid det counterpart, a large reduction in derepressed β-galactosidase activity was detected (section 4.7, Table 4.11). For example det1(pUP39a) displayed 780 units of β-galactosidase activity whereas RHD1(pUP39a), the corresponding heterozygous diploid, displayed only 28 units in the presence of thiamine. As this decrease in derepressed THI4-lacZ expression was associated with the addition of a single copy of the wild-type DET1 gene, it was thought that multiple copies of DET1 carried on a YEp13 vector might result in the complete restoration of the wild-type phenotype. I therefore decided to choose one partially dominant det strain and one recessive det strain for use with this cloning procedure.

The second factor affecting the choice of det strains, was that the cloning strategy was dependent upon the identification of white colonies against a

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background of blue colonies. Therefore the darker the mutant blue colonies the easier it should be to detect the white colonies of interest. As a result the strains det1 and det6 were chosen as these gave the darkest blue colonies of the partially dominant and recessive det strains respectively.

6.3.4 Cloning DET6

Mutant strain det6(pUP39a) was transformed with the YEp13 genomic DNA library and URA+, LEU+ transformants selected on X-gal medium containing thiamine (1.5μM). Approximately 13,000 transformants were screened, from which seventeen potential white colonies were restreaked onto minimal medium lacking leucine and uracil to gain a stock of each. The YEp13 library plasmids within these seventeen candidates were named pLS6.1 to pLS6.17 in order to represent the plasmids from the library screen of det6. β-galactosidase assays were then carried out on cultures of det6(pUP39a, pLS6.1) to det6(pUP39a, pLS6.17) grown in the presence of thiamine (1.5μM). Of the seventeen tested four, shown in bold in Table 6.2, displayed wild-type levels of β-galactosidase activity in the presence of thiamine. These strains were taken on for further analysis to determine whether complementation of det6 by a library plasmid had occurred.

6.3.4.1 Do plasmids pLS6.7, pLS6.10, pLS6.15 and pLS6.16 complement det6?

The det6(pUP39a) strains containing transforming plasmids pLS6.7, pLS6.10, pLS6.15 or pLS6.16 were allowed to lose the URA3 based plasmid pUP39a by growth on non-selective medium. The four ura3 strains were then retransformed with wild-type pUP39a and retested for β-galactosidase activity in medium containing thiamine (1.5μM). All four pUP39a retransformed strains still showed wild-type levels of β-galactosidase activity in the presence of thiamine, indicating that the loss of the det6 mutant phenotype had not occurred as a result of an altered THI4-lacZ reporter gene.

Library plasmid DNA was therefore recovered from the four ura3 plasmid-free det6 strains and transformed into E.coli for amplification, prior to transformation back into the original det6(pUP39a) strain. During this procedure the library plasmid DNAs were examined by restriction enzyme analysis to confirm that they were YEp13 based library plasmids. This was indeed the case, however the genomic DNA inserts in the plasmids were different in all four cases. β-galactosidase assays were then carried out on det6(pUP39a) transformed with each of the four library plasmids in medium containing thiamine (1.5μM) (Table 6.3). From these data it was clear that the four library plasmids did not complement the det6 mutation, as the retransformed det6(pUP39a) strains still displayed the derepressed THI4-lacZ expression phenotype. These results implied
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-gal activity in MM + Thi</th>
<th>Strain</th>
<th>β-gal activity in MM + Thi</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
<td><em>det6</em>(pUP39a, pLS6.8)</td>
<td>4.3</td>
</tr>
<tr>
<td><em>det6</em>(pUP39a)</td>
<td>7.3 ± 2.6</td>
<td><em>det6</em>(pUP39a, pLS6.9)</td>
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<td><em>det6</em>(pUP39a, pLS6.1)</td>
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<td><em>det6</em>(pUP39a, pLS6.10)</td>
<td>0.4 ± 0.2</td>
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<td><em>det6</em>(pUP39a, pLS6.2)</td>
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<td><em>det6</em>(pUP39a, pLS6.11)</td>
<td>5.2</td>
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<tr>
<td><em>det6</em>(pUP39a, pLS6.3)</td>
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<td><em>det6</em>(pUP39a, pLS6.4)</td>
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<td><em>det6</em>(pUP39a, pLS6.13)</td>
<td>5.7</td>
</tr>
<tr>
<td><em>det6</em>(pUP39a, pLS6.5)</td>
<td>5.5</td>
<td><em>det6</em>(pUP39a, pLS6.14)</td>
<td>4.4</td>
</tr>
<tr>
<td><em>det6</em>(pUP39a, pLS6.6)</td>
<td>4.3</td>
<td><em>det6</em>(pUP39a, pLS6.15)</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td><em>det6</em>(pUP39a, pLS6.7)</td>
<td>0.4 ± 0.1</td>
<td><em>det6</em>(pUP39a, pLS6.16)</td>
<td>0.4 ± 0.2</td>
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<td><em>det6</em>(pUP39a, pLS6.8)</td>
<td>4.3</td>
<td><em>det6</em>(pUP39a, pLS6.17)</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Table 6.2 *THI4-lacZ* expression in YEp13 library transformants of *det6*(pUP39a)

β-galactosidase activities of the seventeen white colonies isolated following the transformation of *det6*(pUP39a) with the YEp13 genomic DNA library. Activity levels were compared to W303a(pUP39a) and *det6*(pUP39a) after growth in medium containing thiamine (1.5μM). The four shown in bold type represent the library clones from which complementation may have occurred. The pLS6# plasmids refer to YEp13 based plasmids from the genomic library screen of *det6*. All assays were carried out at least on duplicate samples, and for those shown in bold in triplicate on duplicate samples.
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>det6(pUP39a)</td>
<td>7.3 ± 2.6</td>
</tr>
<tr>
<td>det6(pUP39a, pLS6.7)</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>det6(pUP39a, pLS6.10)</td>
<td>8.4 ± 0.3</td>
</tr>
<tr>
<td>det6(pUP39a, pLS6.15)</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>det6(pUP39a, pLS6.16)</td>
<td>5.0 ± 1.2</td>
</tr>
</tbody>
</table>

Table 6.3 THI4-lacZ expression in det6(pUP39a) retransformed with library plasmids pLS6.7, pLS6.10, pLS6.15 and pLS6.16
β-galactosidase assays were carried out on det6(pUP39a) transformed with the four potential complementing library plasmids, compared to W303a(pUP39a) and det6(pUP39a) grown in medium containing thiamine (1.5μM). All assays were carried out at least in triplicate on duplicate samples.
that the det6(pUP39a, pLS6) strains that originally displayed wild-type THI4-lacZ expression, must have either undergone reversion of the det6 mutation, or gained a second mutation that suppressed the det6 mutation.

No further attempts were made to clone DET6, or the wild-type alleles of other recessive det mutants using the YEpl3 genomic library.

6.3.5 Cloning DET1

Mutant det1(pUP39a) was transformed with the YEpl3 genomic library and transformants selected on X-gal medium lacking leucine and uracil, in the presence of thiamine (1.5µM). Because of the failure in cloning det6, a greater number of transformants were screened to provide a better chance of finding white colonies in which the det1 mutant phenotype was complemented or suppressed. Approximately 75,000 transformants were screened, from which 541 possible white colonies were taken on for further analysis. This number of potential rescuing clones was too many to screen by standard β-galactosidase assays in liquid culture. Therefore an alternative rapid colony-based method was developed in order to identify false positives (figure 6.2). Using this rapid assay, the 541 candidate clones were reduced to two, which were named det1(pUP39a, pLS1.2) and det1(pUP39a, pLS1.69); the pLS1 plasmids refer to the plasmids from the library screen of det1. The two strains were then assayed for β-galactosidase activity quantitatively in liquid cultures containing thiamine (1.5µM), and results shown in Table 6.4.

The two clones, det1(pUP39a, pLS1.2) and det1(pUP39a, pLS1.69), did not show wild-type regulation i.e. complete repression of the THI4-lacZ reporter gene in cultures grown in the presence of thiamine. However they did display a clear decrease in activity when compared to det1(pUP39a). The values of 16 and 17 units of β-galactosidase activity were similar to the 28 units recorded for the heterozygous diploid strain RHD1(pUP39a) under the same growth conditions. This implied that the library plasmids within them might contain the wild-type DET1 allele. To check this the plasmid exchange experiments carried out when attempting to clone DET6, were also carried out with these two clones.

6.3.5.1 Do library plasmids pLS1.2 and pLS1.69 complement det1?

Segregants which had lost the URA3 based plasmid pUP39a were recovered from det1(pUP39a, pLS1.2) and det1(pUP39a, pLS1.69). The ura3 strains were then retransformed with wild-type pUP39a, and retested for β-galactosidase activity in medium containing thiamine (1.5µM); the two new pUP39a transformant clones still showed approximately 20 units of β-galactosidase activity in the presence of thiamine, indicating that their reduction in THI4-lacZ derepressed expression was not due to an altered reporter gene.
Once grown, toothpick a small amount of each colony, including the two controls, into separate eppendorf tubes. Following transformation of det1(pUP39a) with the YEpl3 library, screen for white colonies on X-gal medium containing thiamine. Toothpick 'whites' and det1(pUP39a) and W303a(pUP39a), as positive and negative controls respectively, onto medium containing thiamine (1.5μM). Incubate at 28°C for 2-3 days.

Add 1000μl Z Buffer, 50μl chloroform, 10μl 0.1% SDS to each eppendorf, and vortex for 15". Then add 200μl 4mg/ml ONPG, mix by inversion and incubate at room temperature for 30'.

After 30', stop the reactions by adding 450μl 1M Na₂CO₃ to each tube, and compare their colouration with those of the two controls. The positive control will have turned yellow, whereas the negative control (displaying wild-type regulation) will have remained colourless.

Carry out quantitative β-galactosidase assays in liquid culture on those clones that show wild-type regulation i.e. remain colourless.

**Figure 6.2 Protocol for colony β-galactosidase assay**
Assay designed to identify false positives isolated in the det1(pUP39a) genomic library screen.
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>det1(pUP39a)</td>
<td>781 ± 136</td>
</tr>
<tr>
<td>det1(pUP39a, pLS1.2)</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>det1(pUP39a, pLS1.69)</td>
<td>17 ± 7</td>
</tr>
</tbody>
</table>

Table 6.4 THI4-lacZ expression in det1(pUP39a, pLS1.2) and det1(pUP39a, pLS1.69)

β-galactosidase assays were carried out on cultures of det1(pUP39a, pLS1.2) and det1(pUP39a, pLS1.69) compared to W303a(pUP39a) and det1(pUP39a) grown in medium containing thiamine (1.5μM). All assays were carried out at least in triplicate on duplicate samples. The pLS1# plasmids refer to YEp13 based plasmids from the genomic library screen of det1.

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<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
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</tr>
<tr>
<td>det1(pUP39a)</td>
<td>781 ± 136</td>
</tr>
<tr>
<td>det1(pUP39a, pLS1.2)</td>
<td>626 ± 151</td>
</tr>
<tr>
<td>det1(pUP39a, pLS1.69)</td>
<td>501 ± 32</td>
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</table>

Table 6.5 THI4-lacZ expression in det1(pUP39a) transformed with library plasmids pLS1.2 and pLS1.69

β-galactosidase assays were carried out on det1(pUP39a) transformed with the recovered library plasmids pLS1.2 and pLS1.69. Assays were carried out after growth in medium containing thiamine (1.5μM), compared to W303a(pUP39a) and det1(pUP39a). All assays were carried out at least in triplicate on duplicate samples.
Therefore the pLS1.2 and pLS1.69 library plasmid DNAs were recovered from the ura3 segregants and retransformed into the original det1(pUP39a) strain. Again during the library plasmid recovery and retransformation procedure, the plasmid DNAs were examined by restriction enzyme analysis to check that they were YEp13 based library plasmids. This was the case, but restriction mapping showed that pLS1.2 and pLS1.69 contained different genomic inserts. β-galactosidase assays were carried out on det1(pUP39a) transformed with pLS1.2 and pLS1.69 after growth in medium containing thiamine (1.5μM) (Table 6.5). Both det1(pUP39a, pLS1.2) and det1(pUP39a, pLS1.69) showed β-galactosidase activities comparable to det1(pUP39a), indicating that the retransformed library plasmids did not rescue the det1 mutant phenotype. Therefore as was the case with det6, the det1(pUP39a) library transformants displaying reduced β-galactosidase activities must have been caused by an additional mutation that resulted in reversion or suppression of the det1 phenotype.

6.4 Construction and use of a genomic DNA library from the mutant strain det1

The final strategy employed to clone dominant DET alleles involved the construction of a genomic DNA library from the mutant strain itself. This library would then be transformed into a wild-type DET+ strain, and conferment of the det phenotype onto the wild-type strain screened for as blue colonies on X-gal medium containing thiamine. As the screen was dependent upon the identification of blue colonies against a background of white colonies, the strain det1 was again chosen.

The det1 genomic library, termed pLIB1, was constructed in the same way as the YEp13 library used in section 6.3. Therefore it comprised random genomic DNA fragments obtained by Sau3A partial digestion, cloned into an appropriate plasmid vector. The vector chosen for use in the production of pLIB1 was pEMBLYe30 (Baldari and Cesareni, 1985), as shown in figure 6.3. Plasmid pEMBLYe30 was derived from pEMBL9 (Dente et al, 1983) and contained a number of features that made it suitable for use here. Firstly it contained the yeast selectable nutritional marker gene LEU2, enabling it to be used in conjunction with the URA3 containing plasmid pUP39a. Secondly it contained a unique BamHI restriction site within the α segment of lacZ, into which random Sau3A fragments of det1 genomic DNA could be cloned to produce the library. Also due to the location of the BamHI site, E.coli transformed with plasmids containing DNA inserts could be identified by the blue/white colony colour screen. Finally the plasmid was 2 micron based and therefore multicopy in yeast. This meant that a yeast cell acquiring a plasmid carrying the det1 allele, should definitely form a blue colony on X-gal medium containing thiamine.
Figure 6.3 Plasmid Map of pEMBLYe30.
6.4.1 Construction of pLIB1

Random Sau3A fragments of det genomic DNA in the size range 5-10 kb were to be cloned into the vector pEMBLYe30. According to the formula of Clarke and Carbon (1976) genomic inserts of this size required the production of approximately 6,000 clones to be 95% certain that the library contained a given unique sequence. In order to gain DNA fragments of the required size, a series of pilot digestions involving a range of Sau3A concentrations for increasing lengths of time were performed to determine the necessary conditions. From these reactions an enzyme concentration of 0.1 units/μl and a reaction time of five minutes were decided upon (figure 6.4).

The actual Sau3A partial digestion, a 100 fold scale up of the pilot reaction, was then carried out and DNA fragments greater than 5 kb purified following agarose gel electrophoresis. The det1 genomic DNA was cloned into phosphatase treated pEMBLYe30, transformed into E.coli, and transformants containing recombinant plasmids selected on X-gal and IPTG medium. Plasmid DNA from a number of the white colonies was then isolated and examined by restriction enzyme analysis. This confirmed that the plasmids were pEMBLYe30 based, and that they contained unique det1 genomic DNA insertions of the correct size range. The proportion of recombinant colonies from the E.coli transformation indicated that a genomic library containing approximately 5,000 clones had been produced. Although this number was not as great as the 6,000 clones aimed for, it was large enough to be 90% certain of pLIB1 containing the mutant det1 allele.

6.4.2 Testing the efficiency of the pLIB1 genomic library

The det1 genomic DNA library pLIB1 was tested to determine the frequency of complementation of a recessive auxotrophic mutation, lys2. This experiment checked that the library was functional, and established the frequency at which colonies containing a library plasmid carrying the det1 allele should be detectable upon transformation into W303a(pUP39a). The det1 mutant strain was LYS2, whereas the strain YPH500 carried lys2 and leu2 mutations. Strain YPH500 was therefore transformed with pLIB1 and transformants selected on medium lacking leucine and lysine. As a positive control to calculate the transformation frequency, the transformation mixture was plated onto medium lacking leucine but containing lysine. As a negative control, untransformed YPH500 was plated onto medium lacking leucine and lysine.

The untransformed YPH500 cells gave no colonies on medium lacking leucine and lysine, showing that the leu2 and lys2 markers did not revert. Transformation of YPH500 with pLIB1 DNA gave LYS+ transformants at a frequency of 3 LYS+ / 18,000 LEU+. This indicated that a single gene should be detectable at a frequency of one in every 6,000 transformants using pLIB1.
Figure 6.4 Determination of Sau3A partial digest conditions for det1 genomic library formation

(Lane 1) 1kb ladder, (Lane 2) uncut det1 genomic DNA, (Lanes 3-6) det1 genomic DNA cut with Sau3A (1U/μl) for 5, 10, 15 and 20 minutes, (Lanes 7-10) det1 genomic DNA cut with Sau3A (0.1U/μl) for 5, 10, 15 and 20 minutes, (Lanes 11-14) det1 genomic DNA cut with Sau3A (0.01U/μl) for 5, 10, 15 and 20 minutes
6.4.3 Use of pLIB1 to identify \textit{det1}

The \textit{DET}+ strain W303a(pUP39a) was transformed to leucine prototrophy with pLIB1 DNA and colonies screened for blueness on X-gal medium in the presence of thiamine (1.5\mu M). Approximately 65,000 colonies were screened, from which 27 potential blue colonies were restreaked onto medium lacking leucine and uracil to gain a stock of each. \(\beta\)-galactosidase assays were then carried out on cultures of the 27 candidates, compared to W303a(pUP39a) and \textit{det1}(pUP39a) grown in medium containing thiamine (1.5\mu M). None of the 27 clones tested gave \(\beta\)-galactosidase activity levels greater than 3 units, indicating that they had not acquired a library plasmid carrying \textit{det1}. No further analysis was carried out on these clones or with the pLIB1 genomic library.

6.5 Discussion

In this chapter a number of strategies have been used in attempts to clone wild-type alleles corresponding to both recessive and partial dominant \textit{det} mutations. Although no \textit{det} genes were cloned, it was shown that \textit{det1}, \textit{det2-2}, \textit{det12} and 2-13 were not the result of mutations within either \textit{THI80} or \textit{PDC2}. This result with \textit{THI80} means that if \textit{det1}, \textit{det2-2}, \textit{det12} and 2-13 do contain mutations within a known thiamine gene, then the derepressed expression phenotype they exhibit must be completely novel for that gene. With the recent isolation of the thiamine regulatory genes \textit{THI2} and \textit{THI3} within this laboratory, it will be of interest to see whether plasmids carrying either of these genes are able to restore wild-type regulation when transformed into the \textit{det} strains.

In the YEpl3 based genomic library screens, it was thought that clones carrying \textit{DET1} and \textit{DET6} had been isolated. However upon retransformation of the recovered library plasmids into the original mutant strain, no complementation of the \textit{det} phenotype was observed. As the reporter gene containing plasmid pUP39a was functional in all cases, this implied that the \textit{det} strains had gained a second mutation which caused either reversion or suppression of the \textit{det} mutation. It is clear though that by first testing the efficiency of the YEpl13 library enough transformants were screened, both for \textit{det1} and \textit{det6}, that complementing clones should have been isolated. These clones must therefore have been missed during the X-gal plate screens, despite having used the mutant strains with the clearest phenotype i.e. gave the darkest blue colonies on X-gal medium containing thiamine. As was seen during the original mutagenesis work (section 4.2), the blue colouration of colonies on a given X-gal plate is not uniform making it difficult to distinguish between true white colonies and pale blue colonies. For future library screening of \textit{det} strains, either the X-gal plate assay needs to be modified or a completely different selection
system is required. Such modifications and alternatives will be discussed fully in the general discussion, chapter eight.

The lack of success with the pEMBLYe30 based library pLIB1, made from det1 genomic DNA, cannot be explained by an inability to detect mutant blue coloured colonies upon transformation into W303a(pUP39a). This is because the identification of blue colonies against a background of white coloured colonies has been shown to be possible. 27 candidate blue colonies were detected, although none of these displayed β-galactosidase activity values greater than three units when cultured in liquid medium containing thiamine (1.5µM). This indicated that the blue colony phenotype was not due to a library plasmid containing det1. It is therefore most likely that pLIB1 did not contain a complete random overlapping set of Sau3A fragments from the det1 genome, and in particular did not include one containing det1 itself. Alternatively it is possible that a plasmid carrying det1 did exist, but that such a plasmid was lethal to the cell when present in multiple copies.
CHAPTER SEVEN
ISOLATION AND CHARACTERIZATION OF A MULTICOPY ACTIVATOR OF THIAMINE GENES

7.1 Introduction

Chapter six described the use of a YEpl3 based genomic library in an attempt to clone the DET1 and DET6 wild-type alleles by complementation of their respective mutant phenotypes. An additional use of such 2 micron-based multicopy libraries, is the isolation of genes involved in a given biological process for which no mutations exist (Guthrie and Fink, 1991). An example of this was the isolation of the S.cerevisiae ASK10 as a multicopy activator of SKN7 (Page et al, 1996).

More importantly the S.pombe gene ntf1+ (thi1), was identified as a multicopy activator of the thiamine gene nmt1 (thi3) (Tang et al, 1994; Fankhauser and Schweingruber, 1994). This result was fortuitous as the screen was concerned not with thiamine gene regulation, but instead with identifying factors involved in mitotic initiation; see section 1.4.6. Consequently I decided to carry out a similar study in S.cerevisiae, in the hope of finding multicopy activators of a repressed THI4 gene. This would be carried out by transforming wild-type W303a(pUP39a) with the YEpl3 genomic library, and screening for activators of the THI4-lacZ reporter gene. As in the mutagenesis screen (chapter four), activation of THI4-lacZ was detected by a blue colony colouration on X-gal medium containing thiamine (1.5μM).

7.2 Screening for multicopy activators of THI4-lacZ

The THI+ strain W303a(pUP39a) was transformed with a YEpl3 genomic library (Nasmyth and Tatchell, 1980), and transformants selected on X-gal medium lacking leucine and uracil, but containing thiamine (1.5μM). Approximately 20,000 transformants were screened, from which 24 potential blue colonies were picked onto selective medium to gain a stock of each. β-galactosidase assays were then carried out on liquid cultures of these 24 candidates grown in the presence of thiamine (1.5μM), and the resulting activities compared to those of wild-type repressed cells. Of the 24 tested, only one clone gave derepressed β-galactosidase activity levels greater than 2 units in the presence of thiamine. All subsequent analysis was therefore focused upon this clone, W303a(pUP39a, pWTS4), in which pWTS4 was the YEpl3 library plasmid. The remaining 23 candidates were discarded.
7.2.1 Does plasmid pWTS4 contain a multicopy activator of \( \text{THI4-lacZ} \)?

To check that plasmid pWTS4 caused the derepressed expression of \( \text{THI4-lacZ} \) in the transformant W303a(pUP39a, pWTS4), a \( \text{ura3} \) segregant was recovered which had lost the pUP39a plasmid. The library plasmid pWTS4 was then recovered from the resulting \( \text{ura3} \) strain, and retransformed into W303a(pUP39a). \( \beta \)-galactosidase assays were carried out on both the original and the new W303a(pUP39a, pWTS4) strains after growth in the presence (1.5\( \mu \)M) and absence of thiamine; see Table 7.1.

From these data it was clear that the presence of pWTS4 did indeed result in derepressed \( \text{THI4-lacZ} \) expression, with eight to ten units of \( \beta \)-galactosidase activity being recorded in medium containing thiamine (1.5\( \mu \)M). Furthermore, pWTS4 also resulted in increased \( \beta \)-galactosidase expression in cultures grown without thiamine. Thus it seems possible that pWTS4 carries a gene which can act as a multicopy activator of \( \text{THI4} \), similar to the situation of \( \text{thi1 (ntf1)} + \) in \( S.pombe \).

7.2.2 Identification of ORFs present on pWTS4

In order to determine which genes were present on pWTS4, and therefore candidates for causing the activation of \( \text{THI4-lacZ} \), the genomic DNA insert of pWTS4 was studied by DNA sequencing and restriction mapping. DNA sequence analysis was carried out using the primer KA, which was homologous to a region of the \( \text{Tetr} \) gene immediately flanking the \( \text{BamHI} \) site where genomic DNA had been inserted in pWTS4. At the time of study the complete sequence of the yeast genome had not been released, but the resulting sequence from pWTS4 did find a match to a region already published when used in a FASTA search of the EMBL fungal database. There was a 99.39% match over 328 nucleotides to a region of chromosome IX present on the cosmid clone 8277. Comparisons between the restriction map of cosmid 8277 and that of pWTS4 also indicated that these two vectors carried the same DNA fragment of the yeast genome. From the restriction maps I deduced that the complete pWTS4 genomic insert, which was 5.9kb in length, was not present within cosmid 8277, but that it extended into the overlapping cosmid clone 9150. Figure 7.1 shows the restriction map of the pWTS4 genomic insert, along with the positioning of all ORFs greater than 100 amino acids in length. Information regarding the ORFs was obtained from data describing cosmid clones 8277 and 9150, and by use of the sequence analysis program Gene jockey.

The YEpl3 based library plasmid pWTS4 therefore contained three ORFs which were candidates for the multicopy activation of \( \text{THI4-lacZ} \). The first was \( \text{RPI1} \), which had been previously identified as a multicopy inhibitor of the yeast Ras-cyclic AMP pathway (Kim and Powers, 1991). The second was \( \text{RHO3} \), which
<table>
<thead>
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<td></td>
<td>MM + Thi (1.5μM)</td>
</tr>
<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>W303a(pUP39a, pWTS4)</td>
<td>10.2 ± 2.0</td>
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<tr>
<td>W303a(pUP39a, pWTS4)*</td>
<td>7.8 ± 0.7</td>
</tr>
</tbody>
</table>

Table 7.1 THI4-lacZ expression in W303a(pUP39a,pWTS4)

β-galactosidase activities of W303a(pUP39a, pWTS4) compared to W303a(pUP39a) after growth in the presence (1.5μM) and absence of thiamine. The asterisk denotes the W303a(pUP39a, pWTS4) strain formed by transforming recovered pWTS4 back into W303a(pUP39a). All assays were carried out at least in triplicate on duplicate samples.
Figure 7.1 Restriction map and ORF positioning within the 5.9kb genomic DNA insert of plasmid pWTS4

The 5.9kb of genomic DNA carried in pWTS4 has been drawn with a solid and a dashed section to reflect the regions of DNA present within cosmid clones 8277 and 9150 respectively. Only those ORFs that were greater than 100 amino acids in length have been shown.
belonged to the ras superfamily but had no associated function (Matsui and Toh-e, 1992), and the third was an as yet uncharacterized ORF, YIL117c.

7.2.3 Identification of the ORF responsible for multicopy activation

To identify which of these three ORFs present within pWTS4 was responsible for THI4-lacZ activation, a series of subcloning experiments were carried out. The aim was to gain a set of multicopy plasmids containing solely RPI1, RHO3 or YIL117c, which could then be retested for their ability to activate THI4-lacZ expression in W303a(pUP39a). The first subcloning involved the cloning of RHO3 and YIL117c together, and RPI1 by itself into YEpl3 to produce the plasmids pRB5 and pRB6 respectively; as shown in figure 7.2. The plasmid YEpl3 is identical to YEpl3 except the 2.1kb Xhol-SalI fragment containing the LEU2 gene had religated in the reverse orientation, so destroying both the Xhol and the SalI site, and leaving YEpl3 with a unique SalI site (Broach and Hicks, 1980). This SalI site was used in the formation of pRB5.

Strain W303a(pUP39a) was transformed with pRB5 and pRB6, and β-galactosidase assays carried out on the resultant clones after growth with (1.5μM) and without thiamine. The results of these assays are shown in Table 7.2. The pRB6 transformant displayed 17 units of derepressed THI4-lacZ expression when grown in the presence of thiamine and an enhanced level of expression when grown in the absence of thiamine, indicating that RPI1 was the multicopy activator. In confirmation of this, cultures of the pRB5 transformant which possessed multiple copies of the two other candidate genes, gave only wild-type levels of THI4-lacZ expression.

7.2.4 Do multiple copies of RPI1 affect expression of other TPP-regulated genes?

To test whether multiple copies of RPI1 specifically activated THI4-lacZ or whether they caused a more general effect on all TPP-regulated genes, pRB6 was transformed into W303a strains containing the reporter genes THI5-lacZ (pRB3), THI12-lacZ (pRB4) and THI6-lacZ (pLK2). As a control, pRB6 was also transformed into W303a containing the non-thiamine reporter gene CYC1-lacZ (pUP40). β-galactosidase assays were then carried out on the various strains grown in the presence (1.5μM) and absence of thiamine (Table 7.3).

It was found that the presence of pRB6 activated expression of all three THI-lacZ reporter genes; increased β-galactosidase activities were recorded both in the presence and absence of thiamine. In contrast, pRB6 had no effect upon the level of expression from the CYC1-lacZ reporter construct pUP40. These results therefore implied that the multicopy activation effect of RPI1 was not THI4 specific, but that it was pleiotropic for all TPP-regulated genes.
Figure 7.2 pRB5 and pRB6 production from pWTS4
Table 7.2 Effect of pRB5 and pRB6 on THI4-lacZ expression in W303a(pUP39a)

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
<th>MM - Thiamine</th>
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<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
<td>1060 ± 138</td>
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<tr>
<td>W303a(pUP39a, pWTS4)</td>
<td>10.2 ± 2.0</td>
<td>1574 ± 130</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB5)</td>
<td>1.2 ± 0.2</td>
<td>1114 ± 97</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6)</td>
<td>17.1 ± 2.0</td>
<td>1597 ± 191</td>
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β-galactosidase activity of W303a(pUP39a) grown in the presence (1.5μM) and absence of thiamine, following transformation with pRB5 and pRB6. Activity levels shown are compared to W303a(pUP39a) and W303a(pUP39a, pWTS4) grown under the same conditions. All assays were carried out at least in triplicate on duplicate samples.
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<td>MM + Thi (1.5μM)</td>
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<tr>
<td>W303a(pRB3)</td>
<td>0.6± 0.2</td>
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<tr>
<td>W303a(pRB3, pRB6)</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>W303a(pRB4)</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>W303a(pRB4, pRB6)</td>
<td>10.1 ± 3.2</td>
</tr>
<tr>
<td>W303a(pLK2)</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>W303a(pLK2, pRB6)</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>W303a(pUP40)</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>W303a(pUP40, pRB6)</td>
<td>39 ± 2</td>
</tr>
</tbody>
</table>

Table 7.3 Promoter-*lacZ* expression in strain W303a(pRB6)

β-galactosidase activities of strains W303a(pRB3), W303a(pRB4), W303a(pLK2) and W303a(pUP40) before and after transformation with the *RPI1*-containing plasmid pRB6. All assays were carried out at least in triplicate upon duplicate samples, after growth in the presence (1.5μM) and absence of thiamine.
7.2.5 Does RPI1 act through the normal thiamine-gene activation pathway?

To determine whether RPI1 acted through the normal thiamine-regulation transcription machinery, pRB6 was transformed into the thi2, thi3 and pdc2 mutant strains O58-M5(pUP39a), T49-2D(pUP39a), and UV3(pUP39a) (Nishimura et al, 1992a; Nishimura et al, 1992b; Byrne, pers.comm.). β-galactosidase assays were then carried out on these strains with and without pRB6, grown in the presence of thiamine (1.5μM); see Table 7.4. The plasmid pRB6 did not cause derepressed THI4-lacZ expression in any of the three mutant strains, indicating that the RPI1 activation of THI4-lacZ was indeed dependent upon THI2, THI3 and PDC2, the known thiamine-gene transcription factors.

7.2.6 What is RPI1?

The gene RPI1 has previously been shown to inhibit the Ras-cAMP pathway when overexpressed from multiple copies (Kim and Powers, 1991). The question therefore arose as to how this gene could also effect thiamine gene expression when present in multiple copies. This was particularly true given work carried out in this laboratory which had shown that THI4 expression was not affected by cAMP levels within the cell (Praekelt and Meacock, 1992). Analysis of the Rpi1 protein sequence for known motifs using a "ProSite" search of the EBI database, found only potential sites for phosphorylation and N-myristalisation. The complete protein sequence showed no significant homology to other known proteins, and in particular it was not homologous to the 6-cysteine zinc finger-containing transcription factor thi1, isolated from S.pombe (Tang et al, 1994).

However upon examination of the Rpi1p sequence by eye, it was clear that this protein contained a much greater proportion of asparagine and serine residues than would be expected; of the 407 amino acids, 16% were asparagine, 14 14% were serine, and these residues were grouped together in large runs as indicated in figure 7.3. Homology searches of the EBI database using just these regions identified a number of S. cerevisiae genes that also contained asparagine and serine rich regions, including PPQ (Chen et al, 1993), and FSP1 (Van Aelst et al, 1991). Perhaps more significantly though, PDC2, a gene already shown to be involved in the transcriptional activation of thiamine genes was identified as this too contains clusters of asparagine residues (Richards, 1996; Hohmann, 1993). It was therefore hypothesised that PDC2 might also cause derepressed thiamine gene expression when present in multiple copies. This was however not the case as W303a(pUP39a) transformed with pER1, a YEpl3 based plasmid containing PDC2, displayed wild-type levels of β-galactosidase activity in the presence of thiamine (1.5μM).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a(pUP39a)</td>
<td>+</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6)</td>
<td>+</td>
<td>17.1 ± 2.0</td>
</tr>
<tr>
<td>O58-M5(pUP39a)</td>
<td>thi2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>O58-M5(pUP39a, pRB6)</td>
<td>thi2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>T49-2D(pUP39a)</td>
<td>thi3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>T49-2D(pUP39a, pRB6)</td>
<td>thi3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>UV3(pUP39a)</td>
<td>pdc2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>UV3(pUP39a, pRB6)</td>
<td>pdc2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Table 7.4 *THI4-lacZ* expression in *thi2, thi3* and *pdc2* mutants containing pRB6

β-galactosidase activity of strains W303a(pUP39a), O58-M5(pUP39a), T49-2D(pUP39a) and UV3(pUP39a) before and after transformation by pRB6. All assays were carried out in the presence of thiamine (1.5μM), and are the mean of triplicate assays on duplicate samples.
Figure 7.3 Amino acid sequence of RPI1 gene product
The underlined residues correspond to the main asparagine-serine rich regions present within the Rpi1 protein.
7.3 Disruption of \textit{RPI1}

To verify that \textit{RPI1} was the multicopy activator, and to provide a cassette for use in the disruption of the chromosomal \textit{RPI1} locus, transposon mutagenesis was carried out on the plasmid pRB6 (see experimental procedures section). This resulted in the quasi random insertion of the transposon Tn\textit{HIS3}, a derivative of Tn1000, into pRB6 (Sedgwick and Morgan, 1994). Twenty four tagged plasmids were produced (named pRB6Tn1 to pRB6Tn24) and Southern blot analysis was carried out to determine which contained the transposon within the 3.2kb \textit{HindIII} fragment that encompassed \textit{RPI1}. Figure 7.4 shows the result of hybridising the 0.4kb \textit{XbaI} to \textit{EcoRI} fragment from within \textit{RPI1}, with pRB6 and pRB6Tn1 to pRB6Tn24 digested with \textit{HindIII}. Of the 24 pRB6Tn plasmids tested, the six numbered 6, 8, 9, 10, 14 and 20 (Lanes 8, 10, 11, 12, 18 and 24) no longer possessed a 3.2kb band, indicating that Tn\textit{HIS3} had inserted within this region, and therefore potentially within \textit{RPI1}.

To see how transposon insertion had affected the ability of these six pRB6 derivatives to activate \textit{THI4-lacZ} expression, β-galactosidase assays were carried out on W303a(pUP39a) cultures transformed with pRB6Tn plasmids 6, 8, 9, 10, 14 and 20, grown in medium containing thiamine (1.5 μM); see Table 7.5. W303a(pUP39a) transformed with pRB6Tn9, 10 and 14 displayed wild-type repressed levels of β-galactosidase activity in the presence of thiamine, suggesting that \textit{RPI1} had been inactivated by Tn\textit{HIS3} disruption in these pRB6 derivatives; a fourth clone, W303a(pUP39a, pRB6Tn20), also showed reduced β-galactosidase levels compared to W303a(pUP39a, pRB6). In the remaining two cases, W03a(pUP39a) transformed with pRB6Tn6 or pRB6Tn8, derepressed \textit{THI4-lacZ} expression levels were still observed. However in the case of the pRB6Tn8 transformant, expression levels recorded were more than double those gained for W303a(pUP39a, pRB6). Plasmid pRB6Tn8, along with pRB6Tn10 and pRB6Tn14, were therefore taken on for further analysis.

7.3.1 Tn\textit{HIS3} location within pRB6Tn8, 10 and 14

To determine the exact location of the transposon within plasmids pRB6Tn8, 10 and 14, the DNAs were sequenced using the primer δ which was homologous to bases 86 to 68 within the δ terminal repeat of Tn\textit{HIS3} (Thomas \textit{et al}, 1990). This meant that the sequences gained ran from the transposon out into the pRB6 plasmid, and enabled the point of Tn\textit{HIS3} insertion to be deduced by comparing the sequences produced from each pRB6Tn plasmid with that of pRB6. Figure 7.5 shows the position of Tn\textit{HIS3} within the three plasmids. Whereas the transposon was present within the \textit{RPI1} coding region in pRB6Tn10 and pRB6Tn14, plasmid pRB6Tn8, which showed an even greater derepressed \textit{THI4-lacZ} phenotype than pRB6, possessed the transposon within the region 5' to
Figure 7.4 Southern blot analysis of pRB6Tn plasmids
Analysis was carried out to determine which of the 24 pRB6Tn plasmids contained the TnHIS3 transposon within the 3.2kb HindIII fragment which encompasses RPII. DNA was hybridised with the 0.4kb EcoRI-XbaI fragment from within RPII. (Lanes 1 and 15) uncut pRB6 DNA, (Lanes 2 and 16) HindIII digested pRB6 DNA, (Lanes 3-12) pRB6Tn1-pRB6Tn12 digested with HindIII, (Lanes 17-28) pRB6Tn13-pRB6Tn24 digested with HindIII
<table>
<thead>
<tr>
<th>Strain</th>
<th>β-galactosidase activity in MM + Thi (1.5μM)</th>
</tr>
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<tbody>
<tr>
<td>W303a(pUP39a)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6)</td>
<td>17.1 ± 2.0</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6Tn6)</td>
<td>14.6 ± 3.1</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6Tn8)</td>
<td>38.2 ± 7.1</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6Tn9)</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6Tn10)</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6Tn14)</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>W303a(pUP39a, pRB6Tn20)</td>
<td>2.8 ± 0.9</td>
</tr>
</tbody>
</table>

Table 7.5 THI4-lacZ expression in W303a(pUP39a, pRB6Tn) strains
β-galactosidase activity of W303a(pUP39a) transformed with the six pRB6Tn plasmids in which the transposon is present within the 3.2kb HindIII fragment. All assays were carried out at least twice on duplicate samples, after growth in the presence of thiamine (1.5μM).
Figure 7.5 Location of TnHIS3 insertions within plasmids pRB6Tn8,10 and 14
Diagram showing the position and orientation of TnHIS3 insertion within the 3.2kb HindIII fragment of plasmids (A) pRB6Tn8 (B) pRB6Tn10 and (C) pRB6Tn14. This diagram is not drawn to scale.
This raised the possibility that the insertion had in some way increased the level of RPI1 expression.

7.3.2 Disruption of the chromosomal RPI1 locus

Using the plasmids pRB6Tn8, 10 and 14 it was now possible to replace the chromosomal RPI1 locus with linear DNA fragments containing TnHIS3 either within, or upstream, of the RPI1 coding region. This was achieved by a one-step gene-disruption procedure (Rothstein, 1983), in which homologous recombination occurred between the transformed linear DNA fragment and the chromosomal RPI1 locus. For all three tagged plasmids, the 6kb transposon had inserted within a 1.5kb XbaI to SacII fragment, increasing its size to 7.5kb (figure 7.5). The plasmids pRB6Tn8, 10 and 14 were therefore digested with XbaI and SacII, and the resulting 7.5kb DNA fragments isolated and transformed separately into W303a. Transformants in which integration of the 7.5kb linear DNA fragment into the genome had occurred, were selected on medium lacking histidine. To check that integration had occurred at the RPI1 locus, and not randomly, Southern blot analysis was carried out on genomic DNA isolated from histidine prototrophs generated in the three transformations. Figure 7.6 shows the result of hybridising the 0.4kb XbaI to EcoRI fragment from within RPI1, against XbaI digested genomic DNA from W303a and from W303a transformed with the linear DNA from plasmids pRB6Tn8, pRB6Tn10 and pRB6Tn14. As expected a band of 4kb, comprising the leading 600bp of RPI1 coding sequence and the 3.4kb DNA immediately upstream from RPI1, was seen in the DNA of untransformed W303a (Lanes 1 and 5). In the histidine prototrophs from each transformation (Lanes 2 to 4), this band should shift in size to create a single band of 10kb if the TnHIS3 was present. This was indeed the case, indicating that the one-step disruption procedures had all been successful. With respect to the three pRB6Tn plasmids used to produce these tagged strains, the strains will be referred to as W303aTn8, W303aTn10 and W303aTn14; the latter two carry the rpi1::TnHIS3 allele.

7.4 Phenotypic analysis of the TnHIS3 tagged strains

Previous studies by Kim and Powers (1991) reported that a yeast strain containing an rpi1::LEU2 disruption was sensitive to heat shock. This phenotype was actually based upon the length of time cells took to recover and grow on complete medium following heat shock at 55°C for 20 to 45 minutes. A similar experiment was therefore carried out on strains W303a and W303aTn8, W303aTn10 and W303aTn14. Colonies were replica-plated onto complete medium, heat shocked at 55°C for 0, 10, 20, 30, 40 and 50 minutes, then left to recover at 28°C for 2 days. The exact nature of the phenotype was unclear as no
Figure 7.6 Southern blot analysis of W303a transformants to check for TnHIS3 insertion at the chromosomal *RPII* locus

Genomic DNA from the following strains were digested with *XbaI* and hybridised with the 0.4kb *EcoRI*-*XbaI* fragment from within *RPII*.

(Lanes 1 and 5) W303a, (Lanes 2-4) Histidine prototrophic W303a, isolated following transformation with the 7.5kb *XbaI*-*SacII* fragment from pRB6Tn8 (Lane 2), pRB6Tn10 (Lane 3) and pRB6Tn14 (Lane 4)
data were shown in the Kim and Powers publication, and in my experiment no differences were observed between the wild-type and disrupted strains.

Therefore a new experiment was designed that should detect a thermosensitivity phenotype, if one existed. Strains W303a, W303aTn8, W303aTn10 and W303aTn14 were grown overnight in complete liquid medium and 100µl aliquots added to 900µl volumes of complete medium that had been pre-warmed at 55°C. The cell suspensions were then incubated at 55°C for 0, 20, 40, 60 and 80 minutes, at which times 10µl samples were plated onto complete medium and incubated at 28°C for 2 days. Again no differences were detected in the speed and ability of the four strains to form colonies following the heat shock, indicating that they did not exhibit a thermosensitivity phenotype.

7.4.1 Are W303aTn8, W303aTn10 and W303aTn14 thiamine auxotrophs?

Despite the lack of heat shock phenotype, analysis was still carried out on the three TnHIS3 disrupted strains to determine whether they displayed any phenotypes related to thiamine biosynthesis. To test for thiamine auxotrophy, W303aTn8, W303aTn10 and W303aTn14 were repeatedly subcultured in minimal medium in the absence of thiamine, and growth compared to W303a. All three disrupted strains were able to grow in the absence of thiamine, and at the same rate as the wild-type control. These data therefore indicated that they were not thiamine auxotrophs.

7.4.2 THI4-lacZ expression in W303aTn8, W303aTn10 and W303aTn14

To determine whether RPII was essential for expression of thiamine genes, β-galactosidase assays were carried out on the rpi1::TnHIS3 strains W303aTn10 and W303aTn14, transformed with pUP39a, following growth in the presence (1.5µM) and absence of thiamine. Assays were also carried out on W303aTn8 transformed with pUP39a, as this strain might overexpress RPII due to the TnHIS3 insertion. The results are shown in Table 7.6. All three strains displayed wild-type levels of THI4-lacZ expression in both the presence and absence of thiamine, indicating that RPII was not essential for thiamine gene expression. Furthermore the fact that W303aTn8(pUP39a) displayed wild-type β-galactosidase activity levels implied that in this strain the RPII gene was not expressed to the same level as in W303a(pUP39a, pRB6).

7.4.3 Northern blot analysis of W303aTn8, W303aTn10 and W303aTn14

To verify the THI4-lacZ β-galactosidase activity data in Table 7.5, and to investigate how these related to RPII transcript levels, Northern blot analysis was carried out on RNA isolated from W303a and the three disruptant strains grown in the presence of thiamine (1.5µM). At the same time, RNA was also isolated
Table 7.6 THI4-lacZ expression in W303aTn8, 10 and 14(pUP39a)

β-galactosidase activity of strains W303aTn8(pUP39a), W303aTn10(pUP39a) and W303aTn14(pUP39a) grown in the presence (1.5µM) and absence of thiamine. All assays were carried out at least in triplicate on duplicate samples.
from W303a containing pRB6, pRB6Tn8, pRB6Tn10 or pRB6Tn14, to determine how these plasmids affected RPI1 and THI4 transcript levels in cells grown in the presence of thiamine. RNA was hybridised separately with DNA of the genes (i) ACT1, (ii) RPI1 and (iii) THI4, and the results are shown in figure 7.7.

The RPI1 probe hybridised to two transcripts of sizes 1.7kb and 1.4kb respectively in mRNA isolated from the control strain W303a. As expected the amount of these RPI1 transcripts was greater when pRB6 was present, and even greater still when pRB6Tn8 was present. This result indicated that the TnHIS3 insertion in pRB6Tn8, did indeed cause an increase in RPI1 expression. The corresponding data with THI4 showed that these increases in RPI1 mRNA levels correlated with increases in derepressed THI4 mRNA levels, and that RPI1 was definitely the multicopy activator. Normal THI4 repression by thiamine was seen in both W303aTn10 and W303aTn14, as the transposon insertions in these strains had inactivated RPI1 expression; a shortened RPI1 transcript was detected in W303aTn10, indicating premature termination of RPI1 transcription by the inserted transposon.

Northern blot analysis of RPI1 expression was also carried out on the same eight strains grown in the absence of thiamine, to determine whether RPI1 itself was regulated by thiamine (data not shown). This was not the case, as RPI1 was found to be expressed at the same levels in the presence and absence of thiamine.

7.5 Discussion

Using a YEp13 based yeast genomic DNA library, the gene RPI1 has been identified as a multicopy activator of thiamine genes in cultures grown both in the presence and absence of thiamine. In the case of THI4, Northern blot analysis has shown that this activation is directly proportional to increases in the level of RPI1 mRNA within the cell.

Computer assisted comparison of the RPI1 protein and DNA sequences to those of thi1, the S.pombe multicopy activator of TPP-regulated genes, failed to find any matches. There was however homology to PDC2, a known transcriptional activator of S.cerevisiae thiamine genes, due to the presence of asparagine and serine rich regions (Hohmann, 1993; Raghuram et al, 1994). A number of other transcriptional activator proteins, including S.cerevisiae Pho81p and Dal81p and D.melanogaster mastermind and caudal, also contain a similar 'asparagine rich box' (see figure 1.10). It has been hypothesised that this box plays a direct role in transcriptional activation, although the exact nature of this activation mechanism is as yet unclear (Raghuram et al, 1994). One possibility is that it occurs through protein-protein interactions, in a manner analogous to that of activator proteins containing glutamine-rich regions (Courey and Tjian, 1988).
Figure 7.7 Northern blot analysis of the relationship between $RPI1$ expression and $THI4$ expression

Total cellular RNAs from the following strains grown in the presence of thiamine (1.5μM), were hybridised with probes specific for $ACT1$, $RPI1$ and $THI4$

Lane 1 : W303a  
Lane 2 : W303a(pRB6)  
Lane 3 : W303a(pRB6Tn8)  
Lane 4 : W303a(pRB6Tn10)  
Lane 5 : W303a(pRB6Tn14)  
Lane 6 : W303aTn8  
Lane 7 : W303aTn10  
Lane 8 : W303aTn14
Despite its similarity to \textit{PDC2}, \textit{RPI1} does not appear to activate thiamine genes by either mimicking \textit{PDC2} or by binding to a hypothetical regulatory protein(s) that sequesters Pdc2p when thiamine genes are normally repressed. This conclusion is based upon the observation that \textit{PDC2} did not activate thiamine gene expression when it was present within the cell on a YEpl3 based plasmid, and therefore in multiple copies. Alternative methods for \textit{RPI1} action will be discussed in the final discussion, chapter eight. What is clear though is that \textit{RPI1} acts through the normal thiamine gene transcriptional apparatus, as mutations within any one of the three known thiamine transcriptional activator genes, \textit{THI2}, \textit{THI3} and \textit{PDC2}, abolished the \textit{RPI1} multicopy activation phenotype.

Disruption analysis of \textit{RPI1} using the transposon TnHIS3, found that \textit{RPI1} is not essential for thiamine biosynthesis, or for the wild-type regulation of thiamine biosynthesis. However unlike the reported behaviour of a \textit{rpil::LEU2} strain (Kim and Powers, 1991), the \textit{rpil::TnHIS3} strains did not display a heat shock sensitivity phenotype; Southern and Northern blot analysis confirmed that the strains were disrupted at the chromosomal \textit{RPI1} locus, and that wild-type \textit{RPI1} mRNA was not being produced. It would therefore be interesting to test the Kim and Powers disruption strain for thiamine-related phenotypes, but these strains are not available to us.

An additional finding during the production of \textit{rpil::TnHIS3} disruption strains, was that the plasmid pRB6Tn8 caused an even greater \textit{THI4-lacZ} derepressed activation phenotype than the original pRB6 plasmid. DNA sequencing showed that in pRB6Tn8, the transposon had inserted 5' to the \textit{RPI1} coding sequence, suggesting that TnHIS3 insertion had in some way increased the level of \textit{RPI1} expression. This increase in \textit{RPI1} expression was indeed shown to be the case by comparing \textit{RPI1} mRNA levels in wild-type strains containing pRB6 and pRB6Tn8. Increased expression of neighbouring genes due to read through from transposon based promoters has been observed before. An example of this in \textit{E.coli}, was the activation of \textit{recE} expression from a promoter within Tn5 following nearby insertion (Clark \textit{et al}, 1994). An alternative possibility is that insertion of TnHIS3 within pRB6Tn8 has either disrupted, or blocked, the action of a negative acting regulatory region within the \textit{RPI1} promoter, leading to increased gene expression.

Subsequent to this work it has been shown that like the \textit{S.pombe} gene \textit{thi1}, the \textit{S.cerevisiae} gene \textit{THI2} also encodes a Cys6 zinc finger containing transcription factor. It will therefore be of interest to determine whether this gene also results in the activation of thiamine genes both in the presence and absence of thiamine, when introduced into cells on a multicopy vector.
CHAPTER EIGHT
GENERAL DISCUSSION

This project examined the regulation of thiamine biosynthesis in *S. cerevisiae* in two main ways. Firstly the regulation of known thiamine genes has been studied in various mutant backgrounds, and secondly attempts have been made to isolate and characterise new thiamine regulatory mutations. In the former study, the use of thiamine promoter-*lacZ* reporter constructs showed that *THI4, THI5* and *THI12* display derepressed expression in the *thi80-1* mutant strain T48-2D, in medium containing thiamine. The strain T48-2D possesses reduced thiamine pyrophosphokinase activity, approximately 24% of wild-type, therefore this result indicated that like all previous thiamine genes tested, *THI4, THI5* and *THI12* are also regulated in response to intracellular TPP concentration (Nishimura et al, 1991). This analysis of *THI5*- and *THI12-lacZ* expression also provided the first direct evidence that a gene other than *THI5* from within the *THI5-like* gene family was transcriptionally active; the other family members being *THI11* and *THI13* (Hather, 1996). Prior to the construction of a functional *THI12-lacZ* reporter gene, it had been thought that at least one other member of this gene family was active. This prediction was based upon the lack of sequence divergence within the four *THI5-like* genes, compared to the surrounding chromosomal regions, and the finding that a *thi5::LEU2* disruption strain was prototrophic for thiamine, unlike an *nmt1::ura4* disruption strain in *S. pombe; nmt1* being the sole *THI5* homologue in *S. pombe* (Hather, 1996; Maundrell, 1990). It is interesting to note that comparisons of ORFs present within the sub-telomeric regions of chromosomes containing *THI5-like* genes (figure 1.4), indicate that the chromosome ends VI-L (contains *THI5*) and XIV-L (contains *THI12*) co-evolved, as did X-R (*THI11*) and IV-L (*THI13*). This is seen by the distinct grouping of two different sets of three genes on these two chromosome pairs. Therefore despite being derived from a common ancestor, it will be of interest to see whether the two remaining *THI5-like* genes are also expressed, and if so whether their levels of expression are comparable to that of *THI5* and *THI12*.

Similar assays of *THI4-, THI5-* and *THI12-lacZ* activity in strains carrying *thi2* and *thi3* mutations respectively, indicated that the expression of *THI4, THI5* and *THI12* was dependent upon the transcriptional activator proteins Thi2p and Thi3p. Therefore with the exception of the recently cloned thiamine transport gene *THI7*¹, all thiamine genes tested require *THI2* and *THI3* for transcriptional activation when TPP levels are below repressing (Singleton, in press; Enjo et al, 1997). Recent work in this laboratory has uncovered a third gene required for the transcriptional activation of thiamine genes, namely *PDC2* (Richards, 1996).

¹ In the Enjo et al, 1997 paper, *THI7* is referred to as *THI10*.
Studies have yet to be carried out on PDC2 to determine whether it is required for thiamine transport, and if so whether this requirement is based upon a need to activate THI7 expression.

The second part of this study was carried out to investigate how thiamine genes are deactivated, or repressed, in response to a rise in intracellular TPP. Using a THI4-lacZ reporter gene to monitor THI4 expression, a successful screen was developed for the isolation of both haploid and diploid mutant strains that displayed derepressed expression of THI4-lacZ in the presence of thiamine. Such det mutant strains should potentially comprise mutations within the negatively-acting components of the THI4 transcriptional control system, and as a result further our understanding of thiamine gene regulation. In total 13 det mutant strains were isolated, although following sporulation and tetrad dissection only eight could be shown to contain single gene mutations, and therefore be worthy of further study. Dominance tests of these eight strains indicated that in five cases the det phenotype was caused by a recessive mutation, and in the remaining three by a partially dominant mutation. However the strains were not subdivided according to their dominant or recessive phenotype, but instead upon the β-galactosidase activity levels recorded from cultures of the haploid det mutants grown in medium containing thiamine (1.5μM). Type 1 mutants (det5, det6, det8 and det9) displayed derepressed β-galactosidase activities greater than two units but less than ten units, and type 2 mutants (det1, det2-2, det12 and det2-13) displayed derepressed β-galactosidase activities greater than 600 units. It was the type 2 mutants (including det1, det12 and det2-13 which contained partially dominant mutations) that were of particular interest, as they showed β-galactosidase activity levels in the presence of thiamine comparable to a wild-type strain grown in the absence of thiamine. This level of expression in the presence of thiamine implied that THI4-lacZ regulation had been severely affected, and as a result the remaining analysis focused upon these four strains.

Complementation and meiotic segregation analysis of the four type 2 mutants indicated that they contained mutations within three different genes, these being det1, det2 and det12. Despite being allelic, phenotypic analysis was carried out on both det2-2 and det2-13, as the mutations they contained were clearly different being recessive and partially dominant respectively. By using THI-lacZ reporter genes and Northern blot hybridisations, it was found that the mutations det1, det2-2, det12 and det2-13 were not specific to the regulation of THI4 expression, but that they were pleiotropic for all TPP regulated genes tested. This analysis also showed that the chromosomal THI loci, in addition to their respective reporter genes, were derepressed in the presence of thiamine. Similar observations were obtained following mutagenesis screens looking for derepressed expression of THI5-lacZ and THI6-lacZ (Kew, 1996). Here again
derepressed \( \text{THI-lacZ} \) mutant strains also displayed a pleiotropic effect for all thiamine genes tested. These data, together with the results from the \( \text{thi2}, \text{thi3} \) and \( \text{pdc2} \) mutant strains, imply that TPP regulated genes are controlled by a common set of regulatory factors. The exception to this rule is \( \text{THI17} \) which, although not known to require any gene-specific regulatory proteins, does appear to be activated in a \( \text{THI12} \) independent manner (Enjo et al, 1997). In contrast to a common set of \textit{trans} acting regulatory factors, analysis of thiamine gene promoters has yet to identify any common \textit{cis} acting regulatory sequences. Therefore assuming that thiamine genes are regulated primarily at the level of transcription, these observations would suggest that the relative expression level of each thiamine gene is dictated solely by the ability of the regulatory proteins to bind to the unique gene promoters. In agreement with this theory is the finding from Northern blot analysis that, despite possessing identical transcriptional activators, thiamine genes are expressed at varying levels; \( \text{THI4} \) and \( \text{THI5} \) are both highly expressed, whereas \( \text{THI80} \) is transcribed at a much lower level. Also the \( \text{det} \) mutants isolated in the \( \text{THI4-lacZ} \) mutagenesis screen displayed varying levels of derepression for the other \( \text{THI-lacZ} \) constructs, depending upon which \( \text{det} \) gene was mutant. For example \( \text{det1} \) and \( \text{det12} \) displayed the greatest levels of derepression for \( \text{THI5-lacZ} \) and \( \text{THI12-lacZ} \), whereas \( \text{det2-2} \) and \( \text{det2-13} \) displayed greater derepression of \( \text{THI6-lacZ} \) and \( \text{THI80-lacZ} \). These data supported the view that although the same factors are involved in the regulation of thiamine genes, their ability to bind to a given promoter, and the structure they then form is unique to that gene. Of course one mechanism for increasing expression from a given gene is increased copy number, as could be the case with the \( \text{THI5} \) gene family.

Additional analysis of \( \text{det1}, \text{det2-2}, \text{det12} \) and \( \text{det2-13} \) found that the derepressed expression phenotype was not caused by an inability to (i) synthesise thiamine, (ii) transport thiamine, or (iii) convert taken up thiamine into TPP. All four \( \text{det} \) mutants were prototrophic for thiamine, displayed no obvious defect in thiamine accumulation and did not contain mutations within \( \text{THI80} \). During this examination of thiamine uptake it was observed that despite displaying derepressed expression of thiamine genes, the \( \text{det} \) mutants did not contain increased basal intracellular concentrations of thiamine. This suggested that thiamine biosynthesis is not regulated solely at the level of transcription, but that it must also be subject to a degree of regulation at the level of translation, or enzyme activity. Given the existence of a c-myc tagged \( \text{THI4} \) gene within this laboratory, it will be possible to determine whether \( \text{THI4} \) is regulated at the level of translation by carrying out western blot analysis of \( \text{THI4} \) expression in the \( \text{det} \) mutants, compared to a \( \text{DET}^+ \) wild-type control strain.
The mutant det12 also stood out during thiamine transport analysis, in that it appeared to 'lose' taken up thiamine at a rate much greater than the dilution with growth seen in the control strain or the three other det mutants. As the taken up thiamine was not being excreted back into the extracellular environment an alternative argument is necessary to explain the 'lost' thiamine in det12. One possibility is that the intracellular thiamine was being localised within the cell in such a way as to be unavailable to detection by the thiochrome method employed (Tommasino and Maundrell, 1991). For example the det12 mutation may have resulted in an increased intracellular concentration of a TPP binding protein, or an altered TPP binding protein. However in order to discuss fully the potential genes in which det12 and the other det mutations exist, it is first necessary to devise models which best depict the regulation of thiamine genes in a wild-type strain.

At present it is known that in the absence of exogenous thiamine, the low intracellular TPP levels are non-repressing and thiamine metabolic genes are expressed. In general this expression is dependent upon the action of at least three positively-acting regulatory factors with at least one upstream activation site, such that there is efficient recruitment and formation of the transcription initiation complex at the TATA box (Nishimura et al, 1992a; Nishimura et al, 1992b; Richards, 1996; Nosaka et al, 1992). A simplified version of this binding is depicted in section A, figure 8.1. In this model the three activator proteins have been positioned at the hypothetical thiamine gene promoter, according to knowledge gained from their amino acid sequences. Thus Thi2p which contains cysteine6 Zinc finger domains, a characteristic DNA binding motif (Steitz, 1993), is a good candidate to interact directly with the upstream activation site. In comparison, Pdc2p contains an asparagine-serine rich domain that from preliminary analysis and sequence comparisons, has the potential to act directly as a transcriptional activation domain (Raghuram et al, 1994). Therefore this protein has been placed in contact with the RNA polymerase complex, thereby providing a link between the TATA box and the upstream activation site, via Thi2p. The amino acid sequence of the third regulatory protein, Thi3p, is very interesting in that it contains identical amino acids to those of PDC that are known to bind TPP. This suggests that Thi3p, which is essential for thiamine gene activation, also functions as the protein through which TPP acts in the repression of thiamine gene expression. It has therefore been depicted as a regulatory protein that holds Thi2p and Pdc2p together at the upstream activation site, enabling transcriptional activation to occur. When TPP levels become repressing, TPP binds to Thi3p resulting in a change in conformation, and disruption of the transcriptional activation complex (sections B and C, figure 8.1). To complete the cycle of regulation, thiamine genes would be reactivated.
Figure 8.1 Simple model for thiamine gene regulation
In the absence of thiamine, Thi2p, Thi3p and Pdc2p bind to the upstream activation element enabling recruitment of the RNA polymerase complex and the initiation of thiamine gene expression (A). Upon an increase in intracellular TPP, either by synthesis or transport, TPP enters the nucleus and binds to Thi3p (B). This binding results in a conformational change in Thi3p, its dissociation from the gene promoter, and destabilisation of the transcriptional activation complex (C).
when TPP levels within the cell were no longer repressing, and free Thi3p was again present to stabilise the Thi2p/Pdc2p complex at the upstream activation site. This Thi3p could arise either by TPP being titrated away from Thi3p by another protein, or by the production of new Thi3p. In order to determine whether the latter of these possibilities occurs, it will be of interest to see if the expression of *THI3*, and also *THI2* and *PDC2*, is affected by intracellular TPP concentration.

Having established a working model for thiamine gene regulation, it is now possible to propose domains within proteins that, when mutant, could give rise to a *det* phenotype. However upon carrying out such analysis, it became apparent that if TPP acted directly upon the transcriptional activation complex as shown in figure 8.1, then all mutations leading to a derepressed expression phenotype would be partially dominant! In order for recessive *det* mutations to be explained, the model in figure 8.1 must be modified by the addition of an extra component involved in transporting TPP into the nucleus (figure 8.2). This protein, here referred to as Thi14p, has been depicted as binding to the transcriptional activation complex and not directly to the promoter, upon entry into the nucleus. This is because to date, studies carried out on thiamine gene promoters have yet to identify any potential upstream repression sites. In this study, the complete lack of *det* mutants caused by plasmid-based *THI4-lacZ* promoter mutations also supported the theory that TPP-carrying repressor proteins do not bind to the promoter directly. Once bound to the transcriptional activation complex, TPP is transferred from Thi14p onto Thi3p, leading to a conformational change in Thi3p and the break up of the activation complex, as shown in figure 8.2.

The fact that TPP requires dephosphorylation and an active transport system to cross the external cell membrane also supports the existence of a Thi14-like protein within the cell. Whether Thi14p, or an alternative protein is required to dephosphorylate TPP prior to its transport across the nuclear membrane is as yet unknown. Of course an additional uncertainty in this situation is whether TPP biosynthesis itself occurs within the cytosol or the mitochondria. The c-myc tagged *THI4* gene may therefore also be used to determine where Thi4p is localised within the cell, and so indicate where TPP biosynthesis occurs.

Mutations within *THI14* that result in a recessive *det* mutant phenotype could affect a number of Thi14p domains. The most obvious is the TPP binding domain such that Thi14p has a reduced ability to bind TPP, in the same way as the *thi1* mutation affects aceto-hydroxyacid synthase (Byrne, pers. comm.). Alternatively Thi14p may still be able to bind TPP, but is unable either to translocate into the nucleus, or to bind to the transcriptional activation complex.
Figure 8.2 Advanced model for thiamine gene regulation
In this more advanced model of thiamine gene regulation, Thi2p, Thi3p and Pdc2p interact with the upstream activation site, enabling transcriptional activation to occur by recruitment of the RNA polymerase complex (A). As the intracellular TPP concentration reaches the threshold necessary for repression, the cell is able to sense this by the binding of TPP to Thil4p. This binding alters the conformation of Thil4p such that it is targeted to the nucleus, where it associates with the transcriptional activation complex (B). TPP is then transferred from Thil4p to Thi3p, causing a change in Thi3p structure and the break up of the transcriptional activation complex (C).
once inside the nucleus. Having postulated that strain det2-2 is caused by a mutation within a new gene THI14, the next problem is to explain the allelic partially dominant mutation det2-13. One possibility is that det2-13 results in a defective protein possessing the 'open' Thil4p configuration normally associated with the binding of TPP, even in the absence of TPP. Such a mutant Thil4p would therefore be transported immediately into the nucleus, where it would bind to the transcriptional activation complex. However as no TPP would be delivered to Thi3p, the activation complex would remain intact and derepressed expression would occur. This mutation would be partially dominant because in a det/DET heterozygous diploid strain, the binding of defective Thil4p to the promoter would hinder the subsequent binding of wild-type Thil4p carrying TPP.

None of the four type 2 det mutants contained mutations within PDC2. Therefore assuming no other positively-acting thiamine regulatory factors exist, the two remaining partially dominant mutants, det1 and det12, potentially contain mutations within THI2 and THI3. A possible explanation for the lack of PDC2 mutants is that unlike THI2 and THI3, PDC2 is known to be involved in the regulation of non-thiamine genes e.g. PDC1 and PDC5 (Hohmann, 1993). Therefore mutations within PDC2 that could theoretically cause derepressed thiamine gene expression, might prevent expression of a non-thiamine gene and be lethal to the cell. With regard to the identity of det1 and det12, the 'loss' of taken up thiamine phenotype associated with det12 suggests that it is caused by a mutation within THI3, as this encodes a TPP binding protein. An example of a partially dominant det mutation within THI3 would be one that prevented Thi3p from binding the TPP that had been transported into the nucleus by Thil4p. Alternatively Thi3p may bind TPP, but the TPP induced conformational change does not occur and the transcriptional activation complex remains intact. If the thiochrome method used to determine intracellular thiamine concentration is unable to detect thiamine bound to proteins within the nucleus, this latter mutation could explain the apparent loss of thiamine within det12.

The final type 2 mutant, det1, could be allelic with THI2. Mutations within THI2 that could result in a derepressed phenotype include, those that produce a defective Thi2p that is still able to activate transcription through Pdc2p, despite the conformational change in Thi3p induced by binding TPP. Alternatively det1 may have been caused by an altered Thi2p that obstructs the TPP binding site on Thi3p, thereby preventing transcriptional repression by hindering the transfer of TPP from Thil4p to Thi3p.

As well as providing information about the possible locations of the det mutations, the thiamine regulatory model in figure 8.2 can also explain the isolation of RPI1 as a multicopy activator of thiamine genes. This gene, which has no known function when single copy, has previously been shown to inhibit
the Ras-cAMP pathway when multicopy (Kim and Powers, 1991). However this inhibition is unlikely to be associated with the observed derepression of thiamine genes, because Hather (1996) found that THI4 and THI5 expression was unaffected by changes in intracellular cAMP. Instead it is more likely that the multicopy effect of RPI1 is linked to its homology with PDC2. Both genes contain asparagine-serine rich domains that are thought to be involved in transcriptional activation by their ability to promote protein-protein interactions (Raghuram et al., 1994). One possibility is that Thi14p-TPP binds to the transcriptional activation complex via an interaction with an asparagine-serine rich region of Pdc2p. Therefore Rpi1p, which is thought to be cytosolic, might also interact with Thi14p-TPP due to the presence of its own asparagine-serine rich domains. This interaction could interfere with either the transport of TPP to the nucleus, or the binding of Thi14p-TPP to Pdc2p within the nucleus, such that derepressed expression of thiamine genes occurred. In agreement with this model for RPI1 action are the observations that multiple copies of RPI1 have no effect when THI2, THI3 or PDC2 are mutant, and that an rpi1::TnHIS3 disruption strain has no phenotypes associated with thiamine. One way of testing the model for RPI1 action involves the cloning of THI14, which from analysing the det mutants could potentially be achieved by cloning DET2. The THI14 gene carried on a YEpl3 based vector, could then be transformed into a wild-type strain containing pRB6 and the derepressed expression of THI4-lacZ assayed; pRB6 being the YEpl3 based vector carrying RPI1. As an increased intracellular concentration of Rpi1p might interfere with the action of Thi14p, the simultaneous overexpression of both genes would prevent the multicopy RPI1 activation effect.

If det2 (thi14 ?) and also det1 and det12 are to be cloned, the strategies employed in chapter six need to be improved. With the recent availability of THI2 and THI3 clones within this laboratory, separate plasmids carrying these two genes could be transformed into the det mutant strains, and complementation of the derepressed expression phenotype assayed. From the predicted identities of the det genes, it would be expected that THI2 would complement det1, and THI3 would complement det12. With regard to det2, a candidate gene would be one that encodes a TPP binding protein that is able to be transported from the cytoplasm into the nucleus. One gene that potentially fulfills both these criteria is PDC1. This gene encodes a TPP requiring enzyme that is thought to regulate its own production and the expression of the homologous gene PDC5 (Eberhardt and Hohmann, 1996; Hohmann, 1996). Regulation of PDC5 by Pdc1p occurs at the level of transcription, which is intriguing because PDC5 is also regulated in response to intracellular TPP levels (Richards, pers. comm.). Figure 8.3 summarises our current understanding of PDC and THI gene regulation, and also proposes some additional mechanisms of regulation. In
Figure 8.3 PDC and THI gene regulation
This diagram summarises the positive and negative regulatory mechanisms through which the expression of PDC and THI genes are controlled. It also proposes that Pdc2p is an activator of genes encoding TPP requiring enzymes other than PDC1 and PDC5 (the example shown is PDH), and that Pdc1p is the protein required by TPP to repress gene expression. If Pdc1p is not involved in THI gene repression, then the solid line of repression from TPP to the THI genes must act through an alternative TPP binding protein. This diagram is also hypothetical, in that it is currently unknown whether Pdc1p or Pdc1p-TPP is the repressor of PDC5 expression.
order to determine whether Pdc1p is involved in the regulation of thiamine genes, their expression could be studied in a pdc1Δ strain. If the pdc1Δ strain displayed a det phenotype, then a plasmid carrying the wild-type PDC1 gene could be transformed into det2-2 and det2-13 (and also det1 and det12) to see if complementation occurs. Additional questions raised by figure 8.3 include, why is PDC5 regulated by TPP, are PDC1 and PDC5 derepressed in the det mutant strains, and is Pdc2p needed for the transcriptional activation of other TPP requiring enzymes e.g. PDH? The enzyme PDH has been singled out because PDH and PDC initiate the two pathways by which pyruvate is catabolised within the cell. Therefore if Pdc2p was also an activator of PDH genes, it would be involved in the production of a TPP requiring enzyme irrespective of the growth conditions experienced by the cell. Such a result would imply that Pdc2p was the key protein in the activation of thiamine biosynthetic enzymes, and also genes that encode TPP requiring enzymes. Alternatively, PDC and THI genes may be linked solely for the need to activate PDC5 expression when TPP levels within the cell are low. This is because PDH is believed to have an affinity for TPP that is ten times greater than that of PDC. Therefore when TPP levels are low and PDC is required for the catabolism of pyruvate, increased PDC production might be required to titrate TPP away from PDH.

Should det1, det2 and det12 not be allelic to THI2, PDC1 and THI3 respectively, plasmid-based genomic DNA libraries similar to pLIB1 will have to be constructed in order to clone the partially dominant mutant alleles. However this time the library screen could be improved by either the use of a modified X-gal plate assay, or the use of a THI-reporter gene based not upon lacZ. In a recently described X-gal plate screen, genomic library transformants are plated onto pH 7.0 buffered medium containing thiamine, but lacking X-gal. Once grown, the colonies are then exposed to chloroform for five minutes, overlaid with an agar solution containing X-gal and reincubated at 30°C for 24 hours (Duttwiler, 1996). The proposed advantage of this method is that it does not rely upon cells dying in order for permeabilisation of the cell wall and uptake of X-gal to occur. Therefore the assay is more sensitive and should enable those colonies that have acquired a plasmid carrying a det allele to be detected more easily, as the blue colour they exhibit will be darker. An alternative det cloning strategy could use a THI4-HIS3 reporter gene to monitor THI4 expression. Here a his3, DET strain would be transformed with a genomic library derived from det genomic DNA, and transformants selected for histidine prototrophy in the presence of thiamine. Only those cells that acquire a library plasmid carrying a partially dominant det mutant allele should be able to grow, due to derepressed expression of the THI4-HIS3 reporter gene. These clones can then be transformed with the THI4-lacZ construct to determine quantitatively whether a det allele is present.

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Having cloned the mutant det alleles, it will be necessary to sequence them to determine which genes they correspond to and what the actual mutations are. The information gained from this analysis can be used to test whether the model for thiamine gene regulation shown in figure 8.2 is correct, and if not, how it can be further refined. Additional future work could include the construction and analysis of THI11-lacZ and THI13-lacZ reporter genes, and the analysis of c-myc tagged THI4 gene expression and cellular localisation. The results from the former study will provide further insight into the need for four THI5-like genes within the genome, whereas the latter will tell us about the intracellular location of thiamine biosynthesis and whether thiamine genes are regulated at the level of translation. Finally, figure 8.3 raised a number of questions regarding the overlapping control mechanisms for PDC and THI gene regulation. With our current understanding of these regulatory pathways, it is tempting to speculate that Pdc2p is a global regulator of genes that encode TPP requiring enzymes. Therefore whenever Pdc2p is required to activate gene expression, it also stimulates the genes involved in the production of the cofactor TPP. However if TPP levels within the cell are already sufficient, the proposed intracellular thiamine transport protein Thi14p translocates TPP into the nucleus, whereby it prevents Pdc2p activation of thiamine genes. If Pdc2p is not a global regulator of gene expression, it still appears that despite being a recycled compound required by the cell in only small quantities, the biosynthesis of thiamine is a tightly regulated process that is unlikely to be controlled solely by the concentration of the end product TPP. The extent to which thiamine genes are regulated by factors common to genes encoding TPP requiring enzymes, and whether similar control mechanisms exist for other cofactors remains to be seen.
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