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Research Article

Isolation and characterization of *Saccharomyces cerevisiae* mutants with derepressed thiamine gene expression

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Abstract

Using a *THI4–lacZ* reporter gene, mutant strains have been isolated that display constitutive expression of thiamine genes in the presence of normally repressing levels of exogenous thiamine. In total, eight strains were isolated in which this derepressed expression on thiamine (Det$^-$) phenotype was the result of single gene mutations. The Det$^-$ mutations of three of these strains were partially dominant in a heterozygous diploid configuration, whereas the other five were recessive. The partially dominant mutants *DET1*, *DET12* and *DET13*, and the recessive mutant *det2*, all showed derepressed *THI4–lacZ* expression levels comparable to those of a fully induced normal strain. Use of other promoter–*lacZ* gene fusions revealed that these four mutants were pleiotropic; expression levels of all thiamine-regulated genes tested were also derepressed. Genetic analysis of the four mutants suggested that *det2* and *DET13* were allelic, whereas the others were at different loci; these four mutations therefore represent three different genes. None of the mutations were allelic with *THI80*, mutations of which have previously been shown to confer derepression on thiamine-regulated genes. Also, intracellular thiamine levels were close to normal and none of the four mutants excreted thiamine into the growth medium. All mutant strains were found to be prototrophic for thiamine and none of those tested were compromised for thiamine uptake. It is possible that some may be alleles of, or interact with, the activator gene *THI3*. Taken together, these results imply that *DET1*, *det2*, *DET12* and *DET13* represent new genes encoding negative regulators of thiamine-repressed genes.

Keywords: **thiamine; *THI4*; derepressed mutants; gene expression**

Introduction

As in most plants and micro-organisms, *Saccharomyces cerevisiae* is able to synthesize thiamine (vitamin B_1) *de novo* (Spenser and White, 1997). Biosynthesis occurs via a series of well-documented steps following production of the precursor molecules 4-methyl-5-β-hydroxyethylthiazole (HET) and 2-methyl-4-amino-5-hydroxymethyl-pyrimidine (HMP); for reviews, see Begley (1996) and Estramareix and David (1996). In addition, *S.cerevisiae* is able to acquire thiamine from its extracellular environment using a carrier-mediated active transport system specific for thiamine and its analogues (Iwashima *et al*., 1973, 1975). Thiamine pyrophosphate (TPP), the active form of thiamine, is a recycled enzyme co-factor that does not show high levels of degradation, and the processes of thiamine biosynthesis and transport are therefore tightly regulated to prevent energy wastage by the cell. In the presence of exogenous thiamine, biosynthesis is repressed and the energetically favourable transport system is active. However, as the cell becomes saturated with the vitamin, transport itself is switched off (Praekelt *et al*., 1994). This repression of biosynthesis and transport occurs at least in part at the level of gene transcription. Cells grown in the presence of thiamine display a marked decrease in mRNA levels of all thiamine biosynthetic genes tested (Nosaka *et al*., 1993, 1994; Praekelt *et al*.,

1994), and of the thiamine-repressible acid phosphatase (T-rAPase) encoded by *PHO3* (Schweingruber *et al*., 1986; Nosaka *et al*., 1989). Assays of thiamine biosynthetic enzyme activities have shown that these decrease along with the decrease in mRNA production (Kawasaki *et al*., 1990). Most recently we have discovered that expression of the gene *PDC5*, which encodes an isoform of the TPP-dependent enzyme pyruvate decarboxylase, is also negatively controlled by thiamine (Muller *et al*., 1999).

The key factor in this regulation is the intracellular concentration of TPP, the end product of biosynthesis that is produced directly from thiamine by the *THI80* gene product, thiamine pyrophosphokinase (Nishimura *et al*., 1991).). As in the fission yeast *Schizosaccharomyces pombe*, repression occurs when the intracellular TPP concentration reaches approximately twice its basal level, this being around 9 pmol/10^7 cells (Praekelt *et al*., 1994; Fankhauser *et al*., 1995). Mutations, e.g. *thi80-1*, that reduce thiamine pyrophosphokinase activity lower intracellular TPP levels and lead to derepression of thiamine-regulated genes (Nosaka *et al*., 1993).

The genes *THI2* and *THI3* have been identified as positive transcriptional activators of thiamine biosynthetic genes under non-repressing conditions (Kawasaki *et al*., 1990; Nishimura *et al*., 1992a, b) and *THI3*, but not *THI2*, is also required for activation of the thiamine transporter gene *THI10* (Enjo *et al*., 1997). We have now identified another gene, *PDC2*, as also being required for activation of both thiamine biosynthetic and transport genes (Richards, Byrne and Meacock, unpublished; Hohmann and Meacock, 1998).

In contrast, no genes which actively repress the transcription of thiamine genes, apart from *THI80*, have yet been cloned. Therefore, in order to identify negatively acting components of this system, we have undertaken screens to detect mutants that show derepressed expression of the biosynthetic gene *THI4*. In this paper we report the isolation of both recessive and partially dominant mutations that cause derepression of *THI4–lacZ* expression. These Det$^-$ strains are shown to contain mutations within at least three different genes. Of those tested none appear to be allelic with *THI80*, but some may be allelic with, or interact with, the positive activator *THI3*.

Materials and methods

Yeast strains, culture conditions and plasmids

The Thi$^+$ strains W303a (*MAT**a***, ade2-1, can1-100, leu2-3-112, trp1-1, ura3-1, his3-11-15) and CD1 (MAT**a**/*MATα*, *ade2-1/ade2-1*, *can1-100/can1-100*, *leu2-3-112/leu2-3-112*, *ura3-1/ura3-1*, *his3-11-15/HIS3*) were used for UV mutagenesis. The thi80-1 mutant strain T48-2D (Nosaka *et al*., 1993) was the kind gift of Dr Nosaka, Kyoto University. For the production of strains heterozygous at *DET* loci, Det$^-$ mutants, carrying the *URA3 THI4–lacZ* reporter plasmid pUP39a, were crossed with KBY4 (*MATα*, *ade2-1*, *can1-100*, *leu2-3-112*, *trp1-1*, *ura3-1*, *HIS3*), a derivative of W303α.

All strains were cultured at 30°C in YPD and SD media, made as described by Sherman *et al*. (1986). When defined thiamine concentrations were required, strains were grown in Wickerham's minimal medium (Wickerham, 1951), with thiamine added as stated in Results.

Testing of allele relationships between the Det mutations and the *THI2*, *THI3* and *PDC2* genes was conducted by introducing plasmid-borne wild-type alleles of each gene into the mutant strains. Plasmid pCH1 comprised the *THI3* gene carried on the vector pRS425, plasmid pAW1 comprised the *THI2* gene carried on the vector YEp24, plasmid pER1 consisted of the *PDC2* gene carried on the vector YCp50.

Construction of promoter-lacZ reporter gene plasmids

The *THI4–lacZ* containing plasmid pUP39a was described in Praekelt *et al*. (1994). The other reporter gene plasmids were produced by cloning PCR-derived fragments containing the promoters and initial coding sequences of various genes into pUP34, a YCP50-based plasmid containing the *Escherichia coli lacZ* gene without the eight amino-terminal codons. Thus, all reporter plasmids had a similar architecture (see Figure 1), and carried an in-frame fusion between the inserted gene promoter and its associated open reading frame and the *lacZ* coding sequence. For plasmids pRB3 (*THI5–lacZ*) and pRB4 (*THI12–lacZ*), the *THI5* and *THI12* promoter regions from −667 to +87 were recovered by PCR using pRH11 (Hather and Meacock, unpublished) and pSK1+1 (Van Dyck *et al*., 1995) as templates, respectively. For plasmid pLK3

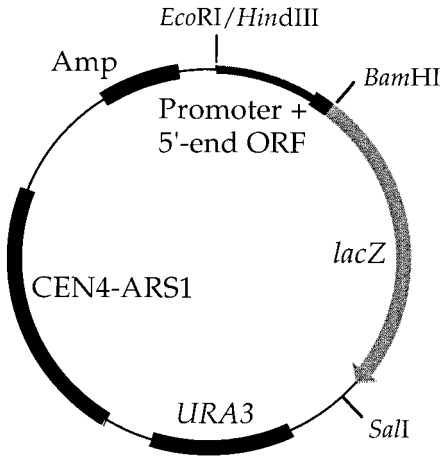

Figure 1. Generic map of lacZ reporter plasmids. Promoter fragments with the start of their open reading frame were inserted between the *Bam*HI and either *Eco*RI or *Hin*dIII sites to give in-frame fusions to the *lac*Z sequence. Details of individual constructions are described in Materials and methods. The plasmid is based upon the YCp50 vector

(*THI80–lacZ*), the *THI80* promoter from −756 to +21 was recovered by PCR using plasmid pAN8 (Nosaka *et al.*, 1993) as a template. The forward and reverse primers (Table 1) used to generate these reporter genes incorporated *Eco*RI and *Bam*HI sites to facilitate cloning into the vector pUP34. In the production of pLK2 (*THI6–lacZ*), the *THI6* promoter region −629 to +33 was recovered by PCR using plasmid pKB16 (Byrne and Meacock, unpublished) as a template. Here the forward and reverse primers (Table 1) incorporated *Hin*dIII and *Bam*HI sites to facilitate cloning. For plasmid pKB39 (*PHO3–lacZ*) the *PHO3* promoter from −763 to +3 was recovered by PCR using genomic DNA of strain S288c as a template. For pKB40 (*THI10–lacZ*) the *THI10* promoter from −658 to +3 was recovered by PCR also using S288c genomic DNA as a template. In these two cases only the ATG of the coding sequences were incorporated into the fusion gene construct, and the forward and reverse primers (Table 1) included *Eco*RI and *Bam*HI sites, respectively, to facilitate cloning. As a control we used an equivalent YCp-type plasmid, pUP40, carrying a fusion of the *lacZ* to the *CYC1* promoter (Praekelt and Meacock, unpublished) which does not respond to thiamine regulation. Finally we used a *PDC5–lacZ* fusion plasmid which can be targeted to integrate at the *URA3* locus and has been described previously (Hohmann, 1993).

Isolation of Det$^-$ mutant strains

UV mutagenesis was carried out using a modified version of the procedure described by Lee *et al.* (1988). Exponential phase cells were harvested, washed in water and resuspended in 0.9% (w/v) KCl. Mutagenesis was carried out by irradiation with a dose of 60 Jm^{-2}. Cells were plated onto minimal medium supplemented with thiamine (1.5 μM) and X-gal (0.12 mM). This medium was made as Wickerham's medium, with the standard Wickerham's salts mixture replaced by M63 salts in order to buffer the medium at pH 6.5 (Clifton *et al.*, 1978). Colonies displaying derepressed *THI4–lacZ* expression were detected by their blue colouration.

Table 1. Oligonucleotide primers used for cloning gene promoters

Reporter plasmid	Gene promoter	Forward/reverse (coordinates)	Nucleotide sequence 5′→3′
pRB3 and pRB4	*THI5* and *THI12*	F (−667 to −650)	GGGGAATTCGTAGAACTAGCGATGCTC
		R (+87 to +70)	GGGGATCCCCGTAACCTTTGGTTTGAGC
pLK2	*THI6*	F (−627 to −608)	GGGAAGCTTCTGGAATCATGATAAGGTC
		R (+35 to +14)	CCCGGATCCAATGAGTAATCAACTTCTTCC
pLK3	*THI80*	F (−756 to –735)	GGGGGGAATTCATGACCATCTTCAGTCGGC
		R (+21 to +2)	CCCCGGATCCTTTTCAATACACTCCTCGCT
pKB10	*THI10*	F (−666 to −646)	CCTGCAAGAATTCTCTCTTGC
		R (+10 to −20)	GGGGATCCATATTGATATAATGCAATTGGC
pKB39	*PHO3*	F (−773 to −753)	GAATACGTTGAATTCATGGGC
		R (+10 to −17)	GGGGATCCATAGGTAATTTGGAATGGC

Primers used in PCR recovery of gene promoters for construction of promoter–lacZ reporter plasmids. Underlined sequences are those complementary to the template DNA. Restriction endonuclease recognition/cleavage sequences were incorporated into the primers to aid cloning of the PCR products.

Sporulation and tetrad analysis

Diploid strains were sporulated by growth on presporulation medium, followed by starvation on sporulation medium (Sherman *et al*., 1986). Upon microscopic detection of tetrads (between 4 and 10 days), asci cell walls were digested with β-glucuronidase (450 units, Sigma) and spores dissected out onto YPD medium using a Singer MSM microdissection system. Segregation of Det^+:Det^- and His^+:His^- phenotypes was determined by growth on X-gal medium containing thiamine (1.5 μM) and SD medium lacking histidine, respectively.

β-Galactosidase assays

Strains were grown to an OD_{600} of approximately 0.5–1.0. Harvested cells were permeabilized with chloroform and SDS, and β-galactosidase activity determined by following the hydrolysis of ONPG (OD_{420}) using the method of Reynolds *et al*. (1989). Units were calculated from the formula:

$$\text{Units} = 1000 \times (\text{OD}_{420})/(t) \times (v) \times (\text{OD}_{600})$$

where t is the reaction time in minutes and v is the reaction volume in ml. The results shown are the average of triplicate assays on a minimum of three independent transformants. In all cases the standard deviation was less than 20%.

Northern blot hybridizations

RNA extraction and Northern blotting was carried out as described previously in Praekelt and Meacock (1990).

Thiamine concentration assays

Measurements of both intracellular and extracellular thiamine concentrations were made using the thiochrome method of Tommasino and Maundrell (1991), as described in Praekelt *et al*. (1994). Thiamine thiochrome fluorescence was estimated in a Shimadzu RF1501 Spectrofluorophotometer at an excitation wavelength of 385 nm and an emission wavelength of 440 nm.

Results

Isolation of strains displaying derepressed *THI4* expression

Strains displaying derepressed expression of *THI4* were isolated from both the haploid strain W303a and, in order to identify dominant mutations, from the diploid strain CD1. To monitor *THI4* expression these strains were first transformed to uracil prototrophy with the *THI4–lacZ* reporter plasmid pUP39a (Praekelt *et al*., 1994, Figure 1). Strains W303a(pUP39a) and CD1(pUP39a) were UV mutagenized, plated onto medium supplemented with X-gal plus a repressing concentration of thiamine (1.5 μM), and the resulting colonies screened for those that displayed derepressed *THI4–lacZ* expression; such colonies were blue due to hydrolysis of the chromogenic substrate X-gal by the expressed β-galactosidase. With strain W303a(pUP39a), 17 000 colonies were screened, of which 37 reproducibly gave blue colonies on X-gal medium containing thiamine. In comparison, of 30 000 colonies screened from the mutagenized diploid strain CD1(pUP39a) only three reproducibly gave blue colonies.

To determine quantitatively whether *THI4–lacZ* expression was derepressed in the 37 haploid and three diploid mutant strains, β-galactosidase assays were carried out on each grown in liquid medium containing thiamine. The expression levels were compared to those of W303a(pUP39a) and CD1(pUP39a) grown in the presence (1.5 μM) and absence of thiamine. Table 2 shows the *THI4–lacZ* expression levels recorded for the 10 mutants isolated from the haploid strain that exhibited the highest derepressed levels and the three diploid mutants. These mutants were named *Det1* to *Det13*, due to their derepessed expression on thiamine. Of the remaining 27 mutants isolated from the haploid strain another 21 also gave β-galactosidase activity values greater than the repressed wild-type strain, although less than those presented in Table 2, but these were not characterized further.

As the mutagenesis screen had involved assay of a plasmid-based reporter gene, mutations resulting in derepressed *THI4–lacZ* expression could be either plasmid or chromosomal in origin. Therefore plasmid-free Ura^- segregants were recovered from mutants *Det1* to *Det10* of the haploid W303a strain and from mutants *Det11* to *Det13* of the CD1 diploid strain. Upon retransformation with fresh DNA of plasmid pUP39a, all 13 strains still displayed derepressed *THI4–lacZ* expression indicating that the Det^- mutations were all chromosomally based.

Table 2. THI4 expression in Det mutants

Strain	Thiamine content of medium (μM)	*THI4* expression level (β-galactosidase activity)
Haploids		
W303a(pUP39a)	1.5	0.5
W303a(pUP39a)	0	1060
W303a(pUP39a) Det1	1.5	781
W303a(pUP39a) Det2	1.5	635
W303a(pUP39a) Det3	1.5	10
W303a(pUP39a) Det4	1.5	7
W303a(pUP39a) Det5	1.5	5
W303a(pUP39a) Det6	1.5	5
W303a(pUP39a) Det7	1.5	5
W303a(pUP39a) Det8	1.5	5
W303a(pUP39a) Det9	1.5	3
W303a(pUP39a) Det10	1.5	2
Diploids		
CD1(pUP39a)	1.5	0.5
CD1(pUP39a)	0	804
CD1(pUP39a) Det11	1.5	16
CD1(pUP39a) Det12	1.5	34
CD1(pUP39a) Det13	1.5	55

Activity of the *THI4* promoter measured by β-galactosidase activities expressed from the *THI4–lacZ* fusion in Det^- mutants of the haploid strain W303a(pUP39a), Det1 to Det10, and of the diploid strain CD1(pUP39a), Det11 to Det13. All cultures were grown in minimal medium containing thiamine (1.5 μM). Values obtained from W303a(pUP39a) and CD1(pUP39a) grown in medium with (1.5 μM) and without thiamine are also shown. The calculation of β-galactosidase units is shown in Materials and methods.

Genetic analysis of Det^- mutant strains

To check whether the Det^- mutants were the result of single gene mutations, the segregation pattern of the Det^- phenotype was monitored for all 13 mutants, following meiosis of Det^-/Det^+ heterozygous diploid strains. For *Det1* to *Det10*, heterozygous strains were formed by crossing each W303a-derived haploid mutant strain with the Det^+ strain KBY4. Diploid strains *Det11*, *Det12* and *Det13* were also tested for heterozygosity at the *DET* loci. Segregation of the chromosomal *his3* and *HIS3* alleles was monitored as an internal control because all diploid strains analysed were originally heterozygous for this locus too. Ura^- meiotic segregants that had failed to inherit the *THI4–lacZ* reporter plasmid were retransformed to uracil prototrophy with DNA of plasmid pUP39a in order to assay their Det phenotype.

The Det^+ control diploid strain CD1 produced meiotic tetrads displaying a segregation pattern of 2:2 for His^+:His^-, and 4:0 for Det^+:Det^-, i.e. all meiotic segregants displayed normal repression of *THI4–lacZ* by exogenous thiamine. Of the 13 heterozygous diploid strains analysed, eight showed 2:2 segregation of both His^+:His^- and Det^+:Det^- indicating that in each case the Det^- phenotype was caused by mutation within a single gene. In the remaining five strains, four repeatedly failed to produce asci giving four viable spores and one, *Det11*, gave unusual segregation patterns for both loci tested. No further work was therefore carried out on these five mutant strains.

Dominance tests were carried out for the eight Det^- mutations exhibiting single gene segregation patterns, by comparing *THI4–lacZ* β-galactosidase expression levels from pUP39a in each mutant haploid strain with those of the corresponding Det^-/Det^+ heterozygous diploid, grown in medium containing thiamine (1.5 μM). Mutants *Det2*, *Det5*, *Det6*, *Det8* and *Det9* were recessive, whereas *Det1*, *Det12* and *Det13* were partially dominant (Table 3). Note that *Det1* was isolated following mutagenesis of the haploid strain, whereas the other two arose from the diploid CD1(pUP39a). The β-galactosidase activities of four of the haploid strains (Table 3) were noticeably higher than normal derepressed *THI4–lacZ* expression values; these being the three partially dominant mutant strains Det1, Det12 and Det13 and the recessive mutant Det2. As these strains each showed a derepressed *THI4–lacZ* expression level similar to that of the wild-type strain grown in the absence of

Table 3. Dominance tests

Mutant	β-galactosidase activity	
	Det^- haploid	Det^-/Det^+ diploid
Det1	781	28
Det2	635	0.8
Det5	5	0.4
Det6	5	0.3
Det8	5	0.4
Det9	3	0.3
Det12	659	34
Det13	1327	55

β-galactosidase activities recorded from haploid Det^- mutant strains compared to their corresponding Det^-/Det^+ heterozygous diploid, following growth in the presence of thiamine (1.5 μM). All strains contained the *THI4–lacZ* reporter gene plasmid pUP39a. The calculation of β-galactosidase units is shown in Materials and methods.

thiamine, it was decided that all subsequent analysis should be focused upon them. The single-gene defects in each were given the designations *DET1*, *det2*, *DET12* and *DET13* in accordance with standard yeast nomenclature (Sherman, 1991).

To determine the number of genes represented by these four mutations, mutant strains of opposite mating types (derived from previous genetic crosses) were crossed against themselves and the three other mutants. Since complementation analysis is not possible with dominant mutations we decided to examine the meiotic segregation patterns of the Det phenotypes from the heterozygous diploids; where necessary, the pUP39a plasmid was transformed into haploid segregants that failed to inherit it during meiosis. We reasoned that allelic mutations would segregate 4:0 for the Det^-:Det^+ phenotypes, thus producing solely parental ditype (PD) tetrads. In comparison, mutations at different loci would generate recombinant Det^+ progeny, as evidenced by the appearance of tetratype (T) and non-parental ditype (NPD) tetrads (Sherman and Wakem, 1991); recombination between two mutations within the same gene would be a relatively rare event. This analysis (Table 4) suggested that the recessive *det2* and the partially dominat *DET13* were allelic, or very closely linked, and that these four Det^- mutant strains could therefore correspond to mutations within three different genes; viz. *DET1*, *DET12* and *det2-2* with *DET2-13*. It is particularly interesting to note that both recessive and partially dominant mutations that confer the same phenotype can arise in the same gene, DET2, viz. *det2-2* and *DET2-13*.

Table 4. Meiotic segregation analysis of Det mutations

Crosses of haploid mutant strains	Number of tetrads with Det^-:Det^+ segregation pattern of: 4:0 (PD)	3:1 (T)	2:2 (NPD)	Mutations at same locus (i.e. allelic?)
Det1 × Det1	9	0	0	Yes
Det1 × Det2	2	6	1	No
Det1 × Det12	2	5	2	No
Det1 × Det13	0	9	0	No
Det2 × Det2	9	0	0	Yes
Det2 × Det12	1	7	1	No
Det2 × Det13	8	0	0	Yes
Det12 × Det12	8	0	0	Yes
Det12 × Det13	1	5	3	No
Det13 × Det13	8	0	0	Yes

Data shown are the segregation patterns of the Det phenotypes within meiotic tetrads of diploids produced from crosses between haploid mutant strains, Det1, Det2, Det12 and Det13. Det phenotypes were assessed by assay of β-galactosidase activity expressed from the *THI4–lacZ* construct on plasmid pUP39a.

All haploid segregants carrying Det^- mutations were also tested for growth on medium lacking thiamine; none were auxotrophic. Therefore these mutations do not inactivate other thiamine biosynthetic genes.

Do Det^- mutations affect all TPP regulated genes?

To test whether the Det^- mutations were specific to the regulation of *THI4*, *ura3* segregants of the Det^+ strain W303a and its *DET1*, *DET12*, *det2-2* and *DET2-13* derivatives were each transformed to uracil prototrophy with the other YCp50-based promoter–*lacZ* reporter plasmids pRB3 (*THI5–lacZ*), pLK2 (*THI6–lacZ*), pKB40 (*THI0–lacZ*), pRB4 (*THI12–lacZ*), pLK3 (*THI80–lacZ*), pKB39 (*PHO3–lacZ*), and a *PDC5–lacZ* YIP-type plasmid. The YCp-type plasmid pUP40, carrying an equivalent *lacZ* fusion to the promoter of the *CYC1* gene which is unaffected by thiamine, was included as control. Assays of β-galactosidase levels were carried out on the various transformed strains grown both in the presence (1.5 μM) and absence of thiamine; the latter tested whether the mutations had any effect on non-repressed expression levels. The data from the W303a transformants (Table 5) clearly confirmed that the *THI4*, *THI5*, *THI6*, *THI10*, *TH12*, *THI80*, *PHO3* and *PDC5* promoters were all repressed by thiamine in the growth medium. In the presence of thiamine, all four Det^- strains also showed derepressed expression of these same promoters, implying that the Det^- mutations were pleiotropic on all thiamine-repressed genes. In contrast, expression levels from the *CYC1* promoter were unaffected by either the presence of thiamine or any of the Det^- mutations (Table 4); we have previously shown, too, that the *PDC1* promoter is unaffected by thiamine (Muller *et al.*, 1999). Therefore, thiamine repression and its alleviation by the Det^- mutations is not a general effect on all yeast promoters. The levels of derepression observed for the other thiamine-regulated promoters in the mutant strains were

generally not as great as that seen for *THI4–lacZ*, which may indicate subtle differences in the regulation of these promoters by the various Det gene products. With the single exception of the *THI10* promoter in the Det12 mutant background, expression levels were higher in cultures grown without thiamine than in those grown with the vitamin. Therefore, these mutations, although relieving the negative control to some extent, do not lift thiamine repression completely.

In addition to assaying expression from plasmid-based reporter genes, Northern blot hybridizations were also used to study chromosomal gene regulation in the Det$^-$ mutant strains. Consistent with the β-galactosidase activity data (Table 5), W303a haploid strains carrying the *DET1*, *det2-2*, *DET12* and *DET2-13* alleles all displayed derepressed transcription of *THI4*, *THI5*, *THI6*, *THI80* and *PHO3* in medium containing thiamine (Figure 2). This supports the conclusion that the Det$^-$ mutations were pleiotropic for all thiamine-repressed genes tested. Our data confirm that *THI80*, encoding the final biosynthetic enzyme thiamine pyrophosphokinase, has a significant basal level of transcription in cells grown with thiamine. This is consistent with a constant need for this enzyme as the only source of the essential co-factor TPP (Nosaka *et al.*, 1993).

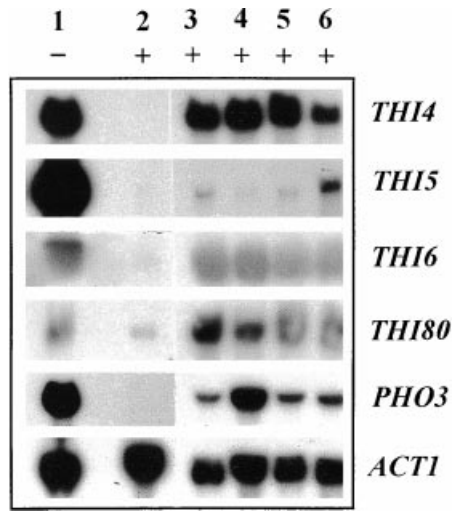

Figure 2. Northern blot analysis of gene expression in Det$^-$ mutant strains. Total cellular RNAs were isolated from the Det$^+$W303a grown in 1.5 μM thiamine (+) or in the absence of thiamine (−) (lanes 1 and 2, respectively), and from the *DET1* (lane 3), *det2-2* (lane 4), *DET12* (lane 5) and *DET2-13* (lane 6) derivatives grown in the presence of 1.5 μM thiamine (+). The blots were hybridized with ^{32}P-labelled probes specific for *THI4*, *THI5*, *THI6*, *THI80* and *PHO3*, as indicated. Hybridization to a probe specific to *ACT1*, which is not regulated by TPP, was included as a control

Table 5. Effect of Det$^-$ mutations on thiamine-regulated genes

	Promoter expression levels (β-galactosidase activities)								
Strain\promoter	***THI4***	***THI5***	***THI12***	***THI6***	***THI80***	***PHO3***	***THI10***	***PDC5***	***CYC1***
+ Thiamine (1.5 μM)									
W303a(pUP39a)	0.5	0.6	0.5	1.0	0.3	18	0.2	0.9	43
W303a(pUP39a) *DET1*	781	51	51	28	6	96	14	11	43
W303a(pUP39a)*det2-2*	635	5	2	49	9	77	5	13	49
W303a(pUP39a)*DET2-13*	1327	10	58	33	4	232	46	43	49
W303a(pUP39a) *DET12*	659	35	19	63	11	59	49	10	42
− Thiamine									
W303a(pUP39a)	1060	428	452	136	30	121	49	77	45
W303a(pUP39a) *DET1*	1406	287	260	158	60	65	44	28	nt
W303a(pUP39a) *det2-2*	1489	110	437	139	45	305	69	105	nt
W303a(pUP39a)*DET2-13*	1554	422	439	193	35	162	72	82	nt
W303a(pUP39a) *DET12*	1150	483	367	159	30	144	45	96	nt

Expression levels of thiamine-regulated promoters in strains carrying Det$^-$ mutations, grown with and without thiamine. Strains were transformed to Ura$^+$ with the YCp50-based promoter–*lacZ* reporter plasmids pUP39a(*THI4*), pRB3(*THI5*), pRB4(*THI12*), pLK2(*THI6*), pLK3(*THI80*), pKB40(*THI10*), pKB39(*PHO3*), pUP40(*CYC1*) and with a *PDC5–lacZ* integrating plasmid (Hohmann, 1993). Promoter activity was estimated by measuring the amount of β-galactosidase produced in the cultures. All measurements were made on triplicate samples of three independent transformants; standard errors were less than 20% in all cases. The calculation of β-galactosidase units is shown in Materials and methods. nt, not tested.

The Det$^-$ mutants are not affected in thiamine uptake

It was possible that *DET1*, *det2-2*, *DET12* and *DET2-13* alleles were mutant forms of thiamine transport genes rather than regulatory genes. Thus, cells grown in medium containing thiamine would be unable to take up the vitamin, and intracellular TPP concentrations would remain non-repressing. To test this, the ability of the haploid Det$^-$ mutants to transport exogenous thiamine was measured and compared to the Det$^+$ W303a (Figure 3). We reported previously (Praekelt *et al.*, 1994), that normal strains completely sequestered 1.5 μM exogenous thiamine from the growth medium within 30 min of its addition; intracellular thiamine levels rose rapidly and then decreased with dilution by increase in culture biomass. The four Det$^-$ strains all transported exogenous thiamine as efficiently as the Det$^+$ parent, indicating that their thiamine transport systems were not compromised. No thiamine was detectable in the growth medium 30 min after addition in all cultures (data not shown), and intracellular levels rose rapidly before falling by growth dilution (Figure 3). However, in the strain carrying the *DET12* allele, intracellular thiamine levels returned to the basal level at a rate somewhat faster than simple growth dilution. This raises the possibility of thiamine degradation or sequestration within the cell, such that it was unavailable to detection by the fluorescent thiochrome method.

To confirm that *DET12* did indeed derepress thiamine gene expression, Northern blot estimation of the *THI4* mRNA level was performed with this strain 1 h after the addition of 1.5 μM exogenous thiamine, and compared to similar experiments with *DET1*, *det2-2*, *DET2-13* and Det$^+$ W303a derivatives. At this time point, all strains contained intracellular thiamine concentrations far in excess of the 20 pmol/10^7 cells necessary for thiamine gene repression (see Figure 3). All four Det mutant strains displayed high levels of *THI4* mRNA 1 h after thiamine addition, indicating that they were indeed derepressed for thiamine gene expression, whereas no *THI4* mRNA was detectable from the Det$^+$ control W303a (data not shown).

None of the mutants excreted excess thiamine into the culture medium (Table 6), even when grown in minimal medium lacking thiamine for extended periods. Additionally the strains contained near normal intracellular amounts of total thiamine, as measured by the thiochrome assay, although mutant *det12* showed a two-fold reduced level (Table 6). However, as we are unable to quantify different thiamine phosphates with this method, we cannot exclude the possibility that the TPP concentration was reduced in all these mutants, and that it is this that caused the derepression phenotype.

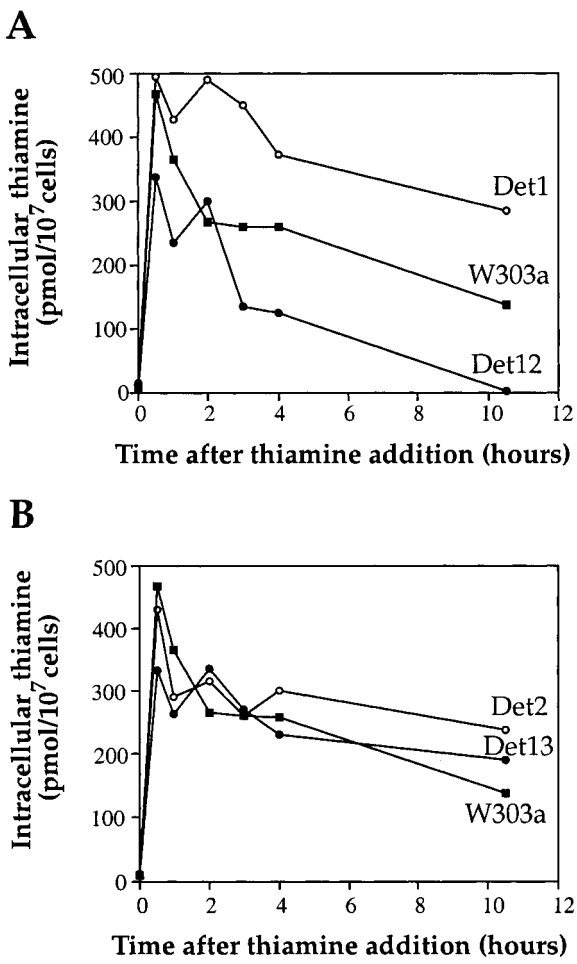

Figure 3. Thiamine uptake in the W303a and the mutant strains Det1, Det2, Det12 and Det13. All strains were grown to 2×10^7 cells/ml in minimal medium lacking thiamine. At time $t=0$, thiamine was added to a final concentration of 1.5 μM. Cell samples were taken at 0, 0.5, 1, 2, 3, 4 and 10.5 h and assayed for intracellular thiamine concentration by the thiochrome method. (A) Thiamine uptake in W303a, and mutant strains Det1and Det12. (B) Thiamine uptake in W303a, and mutant strains Det2 and Det13

Table 6. Thiamine estimations and effect of *THI80* on Det phenotype

	A. Thiamine concentration (pmol/10^7 cells)		**B. *THI4* expression level (β-galactosidase activity)**	
Strain	**Intracellular**	**Extracellular**	**−*THI80***	**+*THI80***
W303a(pUP39a)	8.3 ± 2.4	0	0.5	0.5
W303a(pUP39a)*DET1*	6.8 ± 2.4	0	781	573
W303a(pUP39a)*det2-2*	10.9 ± 2.4	0	635	464
W303a(pUP39a)*DET2-13*	6.3 ± 1.4	0	1327	867
W303a(pUP39a)*DET12*	4.5 ± 1.7	0	659	571
*T48-2D(pUP39a)	nt	nt	5.2	0.5

(A) Intracellular and extracellular thiamine concentrations were measured in cultures grown in thiamine-free minimal medium. Measurements were made by the thiochrome method as described in Praekelt *et al.* (1994). (B) *THI4* expression levels measured as β-galactosidase activities produced by W303a and Det mutant cells carrying plasmid pUP39a, with or without the *THI80* plasmid pRB7. All assays were carried out on cultures grown in the presence of thiamine (1.5 μM). *Strain T48-2D carries the *thi80-1* mutation. The calculation of β-galactosidase units is shown in Materials and methods. nt, not tested.

Are the Det genes allelic with *THI80*?

Previous investigations have shown that mutations which reduce thiamine pyrophosphokinase activity, lead to derepression of the thiamine biosynthetic pathway because of the consequential reduction in the levels of intracellular TPP (Nosaka *et al.*, 1993). These mutations are recessive whereas three of the four Det$^-$ mutations described here show partial dominance. However, since thiamine pyrophosphokinase functions as a homodimeric protein (Nosaka *et al.*, 1993), it is not inconceivable that both recessive and partially dominant mutations could occur that give the same phenotype. Recessive mutations might inactivate the catalytic site of the enzyme, whereas partially dominant mutations might destabilize assembly of the polypeptides into the functional dimer. Therefore, we wished to determine whether any of *DET1*, *det2-2*, *DET12* and *DET2-13* were allelic with *THI80*. Mutant strains were transformed with a YCp-type plasmid, pRB7, carrying the wild-type *THI80* allele, and expression of the *THI4–lacZ* reporter construct plasmid pUP39a was measured. Introduction of the *THI80* allele had no effect; in all four Det$^-$ mutants the derepressed expression of the *THI4* promoter was unaffected (Table 6). Even though three of the mutants are partially dominant, a significant reduction in *THI4–lacZ* expression, equivalent to the levels recorded for the heterozygous diploid strains (Table 3), should have been observed. This was also in clear contrast to the result obtained with strain T48-2D carrying the *thi80-1* allele (Table 6). Here, introduction of the plasmid-borne *THI80* reduced *THI4–lacZ* expression to its normal repressed level; i.e. the strain was restored to a Det$^+$ phenotype. Therefore, we can conclude that neither *DET1*, *det2-2*, *DET12* nor *DET2-13* are alleles of *THI80*.

Are the Det genes allelic with the known positive activator genes?

Previous research from this and other laboratories has identified three genes, *THI2* and *THI3* (Kawasaki *et al.*, 1990; Nishimura *et al.*, 1992a, b) and *PDC2* (Richards, Byrne and Meacock, unpublished; see also Hohmann and Meacock, 1998), which are required for expression of thiamine-regulated genes. We have found (Byrne and Meacock, unpublished data) that mutations in these genes are epistatic on the Det$^-$ mutations; deletion or disruption of each activator gene confers thiamine auxotrophy on all of the Det$^-$ mutants. Thus the derepressed expression on thiamine-regulated genes seen in the Det$^-$ mutants is dependent upon the *THI2*, *THI3* and *PDC2* gene products.

We were interested to ask whether any of the Det genes might be allelic with these activator genes. If so, then subsequent characterization of the mutations would define those parts of the proteins involved in the negative control. To test this, a wild-type allele of each activator gene was introduced into the Det$^-$ mutant strains on a plasmid-based vector and the effects on expression of a *THI4–lacZ* reporter construct monitored. We

reasoned that since the mutations *DET1*, *DET12* and *DET2-13* are only partially dominant in a heterozygous diploid configuration (Table 3), introduction of a copy of a wild-type allele on a plasmid vector should cause a similar phenotypic effect even if not restoring full thiamine repression.

Introduction of the wild-type *THI2* and *PDC2* genes failed to alleviate Det$^-$ phenotypes of strains carrying the *DET1*, *det2-2* and *DET2-13* mutations. In all cases tested, expression of the *THI4–lacZ* reporter construct in cells grown in the presence of thiamine remained high (Table 7). However, when a vector carrying the *THI3* gene was introduced into the Det$^-$ mutants, different behaviour was observed. In three mutants, *DET1*, *det2-2* and *DET12*, expression of the *THI4–lacZ* reporter was markedly reduced relative to the strain transformed with the equivalent vector alone (Table 7), whereas in the *DET2-13* mutant the *THI4–lacZ* expression level was increased over two-fold (Table 7). Since our previous genetic analysis (Table 4) had shown these mutations to lie in three different genes, it is not possible for *THI3* to be allelic with all of them. These data therefore suggest that *THI3* might be allelic with one Det$^-$ mutation and affect the mutant phenotype of the others, or all, in an indirect way. Experiments to investigate the nature of these genetic interactions are currently under way using a gene replacement approach.

Table 7. Effect of additional copies of *THI2*, *THI3* and *PDC2* on Det phenotype

Strain	– (Vector)	+ *THI2* (pAW1)	+ *THI3* (pCH1)	+ *PDC2* (pER1)
DET1	100	101	8	98
det2-2	100	106	29	143
DET2-13	100	149	250	162
DET12	nt	nt	20	118

Percentage expression levels of *THI4–lacZ* measured in Det$^-$ strains carrying plasmid-borne copies of *THI2*, *THI3* or *PDC2* and grown in the presence of thiamine. Because the plasmid vectors used different selectable markers resulting in different growth rates of transformants, the *THI4–lacZ* expresion levels are not directly comparable between mutant strains. Therefore, in each case β-galactosidase levels of activator gene transformants are expressed as percentages of the same strains transformed with the vector alone. Plasmids used are described in Materials and methods. nt, not tested.

Discussion

We report here the isolation of strains that contain mutations within genes required for the transcriptional repression of thiamine biosynthetic genes. The mutants were isolated from both haploid, W303a(pUP39a), and diploid, CD1(pUP39a), strains by their phenotype of derepressed *THI4–lacZ* expression in the presence of exogenous thiamine. Of the 13 mutant strains studied, eight were shown to possess Det$^-$ phenotypes caused by mutations within a single gene. Genetic analysis of these eight found that five of the Det mutations were recessive, and three were partially dominant. This partial dominance was characterized by diploid strains, heterozygous for the *DET* locus, displaying a clearly derepressed *THI4–lacZ* expression phenotype, but at a much reduced level compared to the haploid Det$^-$ counterpart. For example, the mutant *DET1* displayed 781 units of derepressed β-galactosidase activity in a haploid background, compared to 28 units in a heterozygous diploid background (Table 3); the fully repressed level in Det$^+$ haploid and diploid strains was 0.5 units.

Four of the eight single gene mutants were of particular interest, since they displayed derepressed *THI4–lacZ* expression levels almost equivalent to those recorded from a fully induced wild-type strain, i.e. grown in the absence of thiamine. These were the three partially dominant mutants, *DET1*, *DET12* and *DET2-13*, and the recessive mutant *det2-2*. A series of crosses and meiotic segregation analyses suggested that mutations *det2* and *det13* could be alleleic, and that the four strains corresponded to mutations within three genes, defined as *DET1*, *DET2* and *DET12*. These four strains were prototrophic for thiamine biosynthesis, exhibited wild-type thiamine uptake, and were not alleles of the thiamine pyrophosphokinase gene *THI80*. This implied that in the presence of 1.5 μM exogenous thiamine, intracellular TPP levels should be repressing and therefore the Det$^-$ phenotypes were the result of mutations within negatively-acting regulatory factors.

Using a range of promoter–*lacZ* reporter constructs and Northern blot hybridizations, it was shown that the mutations in *DET1*, *det2-2*, *DET12* and *DET2-13* were not specific to the regulation of *THI4*, but were pleiotropic for all thiamine-regulated genes tested; the Det genes normally function in transcriptional repression of thiamine

genes. However, although expression of all these thiamine-regulated genes was derepressed in these mutants, the strains did not overproduce the vitamin or excrete it into the medium. This must mean that other levels of regulation, perhaps feedback inhibition of enzyme activities, also act to control the biosynthetic pathway.

How TPP, which normally functions as a co-factor of cytosolic and mitochondrial enzymes, mediates control of nuclear genes is unclear. Since our mutagenesis regime failed to generate *cis*-acting dominant derepressed mutations within the *THI4* promoter sequence of plasmid pUP39a, we surmise that the negative control is unlikely to act directly through protein–DNA interactions. More likely it acts through protein-protein interactions which modulate the activity of a positive transcriptional activator which does recognize the promoter sequence. Presumably TPP causes the alteration of a protein that enters the nucleus to effect the control; the Det$^-$ mutations could be in structural genes encoding a signalling protein or its transcription factor target. Therefore a key question now remains as to which genes carry the Det$^-$ mutations.

Recently, the mutant strain *thi81* was isolated which also displays constitutive thiamine metabolism in the presence of normally repressing levels of exogenous thiamine (Nishimura *et al*., 1997). That mutant also had intracellular thiamine and thiamine phosphate levels similar to wild-type. It is impossible to say at this stage whether any of the Det$^-$ mutations reported here are allelic with *thi81*. That mutation is recessive, whereas three of the Det$^-$ mutations are partially dominant. Nevertheless, it would be of considerable interest to determine the relationship between them.

To date, three positive activators of thiamine metabolism genes have been identified. These are *THI2* (ORF YBR240c) encoding a zinc-finger protein, *THI3* (ORF YDL080c) encoding a Pdc-like protein reported to have α-ketoisocaproate decarboxylase activity (Dickinson *et al*., 1997), and *PDC2* (ORF YDR081c) encoding a protein rich in serine and asparagine residues (see Hohman and Meacock, 1998). If the Det$^-$ mutations lie in any of these genes, then they must alter the encoded protein such that its function is no longer sensitive to elevated intracellular TPP levels. Of these three only Thi3p appears to contain a TPP binding domain. We have reported here that the introduction of extra copies of the wild-type *THI3* gene affects the phenotype of all four Det$^-$ mutants (Table 7). Therefore it seems possible that it is indeed this protein that is responsible for transmitting the TPP signal and experiments to investigate this in detail are currently under way. However, since the Det$^-$ mutations are apparently located at three separate loci (Table 4), there must be at least two more proteins involved in this signal transmission and transcriptional repression of thiamine genes when intracellular TPP levels are repressing.

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