Isolation and Characterization of Regulatory Mutants From *Schizosaccharomyces pombe* Involved in Thiamine-Regulated Gene Expression

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ABSTRACT

Mutants from *Schizosaccharomyces pombe* deficient in the regulation of thiamine-repressible acid phosphatase have been isolated. Mutants expressing derepressed levels of the enzyme in the presence and absence of thiamine map in three genes, *tnr1*, *tnr2* and *tnr3*. mRNA levels of the *pho4* gene (coding for thiamine repressible acid phosphatase) and another thiamine-regulatable gene, *thi3* (coding for a thiamine biosynthetic enzyme and corresponding to *nmt1*) are constitutively synthesized in the mutants. The mutants also exhibit constitutive thiamine transport which is thiamine repressible in wild type. The *tnr3* mutants reveal a 10–20-fold higher intracellular thiamine level than *tnr1* and *tnr2* mutants and wild type. Mutants expressing repressed levels of thiamine-repressible acid phosphatase map in gene *thi1*. No or little amounts of *pho4*- and *nmt1*-specific mRNA can be detected. These mutants are impaired in thiamine uptake and are thiamine auxotrophic due to the inability to synthesize the thiazole moiety of the thiamine molecule. All tested *tnr* and *thi1* alleles are recessive, and *thi1* mutations are epistatic over *tnr* mutations. We assume that the *thi1* and *tnr* genes are involved in thiamine-mediated transcription control.

THIAMINE (vitamin B_1) regulates gene expression in yeast. In *Schizosaccharomyces pombe* it represses mRNA synthesis of *pho4*-coded acid phosphatase and of a gene product coded for by gene *nmt1* (M. E. SCHWEINGRUBER *et al.* 1986; MAUNDRELL 1990). Both genes control thiamine metabolism. *Nmt1* corresponds to gene *thi3* and is responsible for the synthesis of the pyrimidine moiety of the thiamine molecule (SCHWEINGRUBER *et al.* 1991). Thiamine-repressible acid phosphatase is a *N*-glycosylated cell wall protein and is believed to dephosphorylate thiamine phosphates which may occur as natural substrates in growth media (M.E. SCHWEINGRUBER *et al.* 1986; SCHWEINGRUBER *et al.* 1991). We also observed that thiamine represses thiamine transport and mating in fission yeast (SCHWEINGRUBER *et al.* 1991; SCHWEINGRUBER and EDENHARTER 1990). Whether this is achieved by regulated gene expression still has to be shown. For *Saccharomyces cerevisiae* repression of a gene *pho3* which corresponds to thiamine-repressible acid phosphatase in *S. pombe* has been observed by M. E. SCHWEINGRUBER *et al.* (1986) and confirmed by NOSAKA *et al.* (1988).

The role of vitamin B_1 as coenzyme (in form of its diphosphate) in different reactions of the carbohydrate metabolism has been known for a long time (for review see FRIEDRICH 1987). The first observation that thiamine can also regulate gene expression has been reported only a few years ago (M. E. SCHWEINGRUBER *et al.* 1986), and the role of the vitamin as regulator of gene expression has not yet been explored. As a step toward this goal we report in this communication isolation and characterization of mutants from *S. pombe* defective in thiamine-regulated gene expression.

MATERIALS AND METHODS

Strains and media: *S. pombe* wild-type strains containing the *mat2* and nutritional markers and acid phosphatase-deficient mutants are from our collection; strain *pho1-44* has the entire *pho1* gene coding for phosphate-repressible acid phosphatase deleted (ELLIOT *et al.* 1986). Strains were grown in supplemented or unsupplemented liquid (MM) or solid (MMA) minimal medium as described (SCHWEINGRUBER and EDENHARTER 1990) or in liquid (YE) or solid (YEA) yeast extract medium.

Determination of acid phosphatase activity: Activity was tested on plates with α-naphtyl phosphate and Fast Blue (SCHWEINGRUBER, SCHWEINGRUBER and SCHUEPACH 1982) and in liquid cultures by *p*-nitrophenyl phosphate (A. M. SCHWEINGRUBER *et al.* 1986).

Isolation and mapping of *tnr* and *thi* mutants: Equal amounts of cells from strains *pho1-44h*$^+$ and *pho1-44h*$^-$ were mixed and mutagenized with nitronitrosoguanidine (NNG) as described by DHAMIJA, FLURI and SCHWEINGRUBER

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(1986). Cells were sporulated and the spore suspension was used as the source to isolate *thi* and *tnr* mutants. *tnr* mutants were obtained by staining colonies on YEA plates for acid phosphatase activity. Red colonies (acid phosphatase activity is not repressed) were picked. The nmol *thi* mutants were isolated on MMA plates containing 40 nm thiamine per liter. They stained only very weakly with the acid phosphatase plate assay. Out of roughly 40,000 tested colonies 20 *tnr* and 8 *thi* mutants could be isolated. The mutants were back-crossed and subsequently tested for allelism by crossing them with each other and by counting wild-type recombinants essentially as described by SCHWEINGRUBER *et al.* (1991).

Construction and analysis of diploids and double mutants: Standard genetical methods were used (GUTZ *et al.* 1974). For the construction of stable diploids heterozygous or homozygous at the *tnr* or *thi1* loci haploid *tnr* and *thi1* strains in a *pho1-44 ura4-D18* background were crossed with a *pho1-44ade7-50mat2-102* strain containing the mutant or wild-type allele of the genes in question. The *mat2-102* allele allows the maintenance of stable diploids (EGEL 1984). Diploids were tested for acid phosphatase activity on plates and in liquid cultures. *thi1* diploids were also tested on plates and in liquid medium for thiamine auxotrophy. To obtain *tnrthi1* double mutants, *tnr* mutants were crossed with *thi1* strains and double mutants were isolated from asci of the recombinant ditype. They were assayed for acid phosphatase activity and thiamine auxotrophy as the diploid strains.

Growth experiments with *thi* mutants: Requirement of *thi* mutants for thiamine or intermediates of thiamine synthesis was tested in growth experiments as described before (SCHWEINGRUBER *et al.* 1991). 4-Amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole (later referred to as pyrimidine and thiazole moieties) were kindly supplied by G. MOINE from Hoffmann-La Roche and Co. AG, Basel.

Extraction and determination of thiamine and thiamine phosphates: Thiamine and its phosphates were extracted in HCl and determined by high performance liquid chromatography as described previously (SCHWEINGRUBER *et al.* 1991).

Determination of thiamine uptake: Cells were grown in MM or MM containing thiamine to an optical density of 2–4 at 530 nm, washed and tested for thiamine uptake as described by SCHWEINGRUBER *et al.* (1991).

Northern blot hybridization: Strains were grown in MM, MM containing 1 μM thiamine or YE to a density of 1–2 × 10^7 cells and RNA was extracted as described by GRIMM *et al.* (1991). RNA was separated on 1.2% agarose/glyoxal gels, transferred to GeneScreen membranes (NEN) with a vacuum blotter (Pharmacia) and hybridized to ^{32}P-labeled *ura4*, *pho4* and *nmt1* probes by the dextran sulfate method. Glyoxal gels and hybridization were done according to the GeneScreen manual. The *ura4* signal is weaker than the *nmt1* and *pho4* signals and required longer exposure time.

RESULTS

Isolation of repressed and derepressed mutants for thiamine-repressible acid phosphatase: Acid phosphatase of *S. pombe* is coded for by two genes, *pho1* and *pho4*. *pho1* is repressed by phosphate and *pho4* by thiamine (for review, see SCHWEINGRUBER 1987). To obtain mutants defective in thiamine-regulated *pho4* expression, we mutagenized a strain which has the *pho1* gene deleted (*pho1-44*) and isolated by a plate assay diagnostic for acid phosphatase activity mutant colonies exhibiting aberrant thiamine-repressible acid phosphatase activity in response to the thiamine signal.

TABLE 1

Acid phosphatase activity of *tnr* and *thi1* mutant strains grown in different media

Strain	Acid phosphatase activity[a] from cells grown in		
	MM	MM + thiamine[b]	YE
pho1-44	100	7	5
pho1-44thi1-1	8[c]	6	8
pho1-44thi1-3	12[c]	11	13
pho1-44thi1-23	9[c]	8	11
pho1-44tnr1-9	135	71	40
pho1-44tnr1-13	136	71	128
pho1-44tnr1-18	128	70	168
pho1-44tnr2-2	100	35	131
pho1-44tnr2-11	114	29	110
pho1-44tnr3-5	121	71	280
pho1-44tnr3-8	128	107	210
pho1-44tnr3-10	150	121	321

[a] Given as percentages of the activity of the parent strain grown in MM.
[b] Concentration of thiamine is 1 μM.
[c] MM contains 40 nmol thiamine per liter.

To isolate mutants derepressed for *pho4*-coded acid phosphatase activity, mutagenized cells were grown on MM containing repressing amounts of thiamine, and mutant colonies with derepressed acid phosphatase levels were looked for. No mutants with fully derepressed levels could be selected. Previously we have shown that the only component in yeast extract which is responsible for repression of *pho4*-coded acid phosphatase is thiamine (M. E. SCHWEINGRUBER *et al.* 1986). We therefore also screened for thiamine nonrepressible mutants on YEA plates and isolated over 20 mutants with high activity. The mutants were tested for allelism and two to two segregation. They map in three genes, *tnr1* (12 mutants), *tnr2* (2 mutants), and *tnr3* (6 mutants). The genes are unlinked. Acid phosphatase activity of the mutants was quantitated after growth in YE and MM containing no or 10 μmol of thiamine per liter. In YE acid phosphatase of the mutants is not repressed; in contrast, it is stimulated, particularly in *tnr3* mutants. In MM thiamine still has some repressing effect on acid phosphatase activity. The results for representative mutants are given in Table 1.

In a second set of experiments we plated cells on MMA and isolated mutants expressing no or little acid phosphatase activity. (To prevent missing of pleiotropic mutants which are also repressed for thiamine biosynthesis, nonrepressing amounts of thiamine were added to the MM.) Sixteen mutants were isolated.

TABLE 2

Growth of *thi1* mutants

Strain	Growth on MM supplemented with			
	—	Pyrimidine	Thiazole	Thiamine
pho1-44	8.95	8.55	8.6	8.4
pho1-44thi1-1	0.1	0.2	7.1	8.2
pho1-44thi1-23	0.2	0.2	7.0	8.2

Strains were grown in MM containing either thiamine or the indicated intermediates of thiamine biosynthesis at a concentration of 0.35 μM for 27 hr at 30°. Growth was determined by measuring the optical density at 530 nm.

TABLE 3

Intracellular levels of thiamine diphosphate (TDP), thiamine monophosphate (TMP) and thiamine in *tnr* mutants

Strain	TDP[a]	TMP[a]	Thiamine[a]
pho1-44	6.7	1.0	0.8
pho1-44tnr1-1	8.2	1.4	0.7
pho1-44tnr1-12	7.5	1.3	0.8
pho1-44tnr2-2	7.4	1.4	1.1
pho1-44tnr2-11	7.1	1.2	1.2
pho1-44tnr3-8	5.3	0.8	12.2
pho1-44tnr3-10	5.9	0.9	13.5

Cells were grown in MM, and thiamine and its phosphates were extracted and determined as described in MATERIAL AND METHODS.

[a] Given as pmol per 1 ml cells with an optical density of 1 at 530 nm.

They were assayed for two to two segregation, tested quantitatively for acid phosphatase activity and examined for allelism with each other and with *pho4* mutants. Ten mutants were allelic to *pho4*. Six mutants mapped in a gene called *thi1* (which is not allelic to the *tnr* genes) and exhibited about 10% of the parent strain acid phosphatase activity when cultivated in MM. The residual activity of the mutants could not be repressed anymore by adding thiamine to the MM or by thiamine present in yeast extract. Acid phosphatase of these mutants is apparently permanently repressed. The data of three representative mutants are given in Table 1.

Intracellular thiamine levels of *thi1* and *tnr* mutants: Knowing that the expression of at least one gene responsible for thiamine biosynthesis (*nmt1* which is allelic to *thi3*) is thiamine repressible, we tested all our mutants for thiamine auxotrophy and intracellular thiamine levels. The results are shown for two representative mutants of each gene in Tables 2 and 3. The *thi1* mutants are auxotrophic for thiamine. The thiamine molecule consists of pyrimidine and a thiazole moiety. The two halfs of the molecules, 4-amino-5-hydroxymethyl-2-methylpyrimidine and 4-methyl-5-(2-hydroxyethyl)thiazole, are synthesized in separate pathways and joined together to thiamine monophosphate (for review, see YOUNG 1986). To elucidate the biosynthetic step in which *thi1* mutants

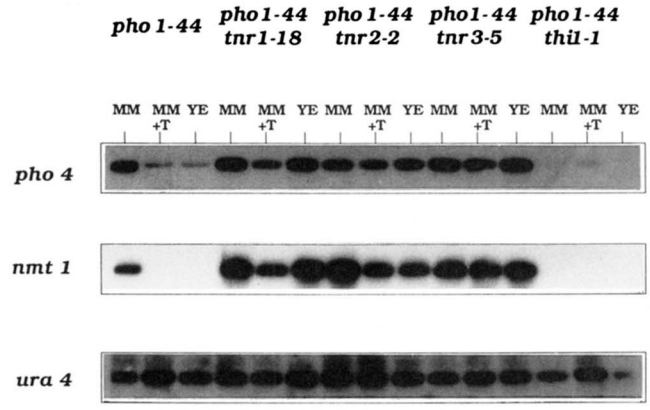

FIGURE 1.—Northern hybridization analysis of *pho4* and *nmt1* mRNA of *tnr* and *thi* mutants. RNA from cells grown in MM, MM containing 1 μM thiamine (MM+T) or YE was extracted and probed with the two thiamine-regulatable genes *pho4* and *nmt1* as described in MATERIAL AND METHODS. As a control the RNA was also probed with gene *ura4* which is not regulated by thiamine.

are defective they were grown in the presence of thiazole, pyrimidine and thiamine. The mutants do not grow in MM unless it contains thiazole or thiamine (Table 2). This indicates that the *thi1* mutants are unable to synthesize the thiazole moiety. The *tnr* mutants are prototrophic for thiamine and the *tnr1* and *tnr2* mutants have normal intracellular thiamine levels. The *tnr3* mutants reveal intracellular levels of unphosphorylated thiamine that are more than a factor of 10 higher than in the parent strain (Table 3). Similar to the *thi1* strains, these mutants are apparently not only defective in the expression of thiamine-repressible acid phosphatase but also in the regulation of the intracellular thiamine pool.

Northern hybridization analysis of *pho4* and *nmt1* mRNA in *tnr* and *thi1* mutants: So far the only thiamine-regulated genes reported are *pho4* and *nmt1*. The cloned genes were used as probes to measure specific mRNA levels from cells grown in MM, MM containing thiamine, and YE extract medium. The results are shown in Figure 1. They confirm previous reports that the expression of the two genes is thiamine repressible and that repression is stronger for *nmt1* than *pho4*. They show also that mRNA levels of the two genes are derepressed under all tested growth conditions in *tnr1*, *tnr2* and *tnr3* mutants. As already observed for the enzymatic activity, *pho4* mRNA is not fully derepressed in cells grown in MM containing thiamine but is high, particularly in *tnr3* mutants, in cells cultured in YE. In the *thi1* mutants *pho4* and *nmt1* mRNA levels are repressed.

Thiamine transport in *thi1* and *tnr* mutants: We have previously shown that thiamine transport is a thiamine-regulated process (SCHWEINGRUBER *et al.* 1991). Pregrowth of cells in MM containing for example a concentration of 1 μM thiamine reduces thiamine uptake rates 5–10-fold. To examine whether

TABLE 4

Thiamine uptake in *tnr* and *thi1* mutants

Strain	[^{14}C]Thiamine uptake from cells pregrown in	
	MM	MM + 1 μM thiamine
pho1-44	100[a]	12
pho1-44tnr1-9	120	89
pho1-44tnr1-18	122	93
pho1-44tnr2-2	100	44
pho1-44tnr2-11	95	51
pho1-44tnr3-8	126	112
pho1-44tnr3-10	125	102
pho1-44thi1-1	16	2
pho1-44thi1-3	34	2

Strains were grown in the presence and absence of thiamine and subsequently assayed for thiamine uptake as described in MATERIAL AND METHODS. Uptake of thiamine was measured for 2 hr. Values are given as percentages of the uptake of the parent strain *pho1-44* pregrown in MM.

[a] Absolute value is 6951 cpm/0.5 × 10^7 cells.

mutations in *tnr* and *thi1* genes also affect thiamine transport we tested several mutants of each gene for thiamine uptake. The data for mutants mapping in the same gene were always very similar. The results for two mutants of each gene are given in Table 4. MM-grown *tnr* mutant cells exhibit similar uptake rates as the parent strain. Down-regulation of thiamine uptake is, however, only weak in the mutants pregrown in MM containing thiamine. *Thi1* mutants reveal significantly lower uptake rates than the parent strain but uptake can still be repressed by a similar factor as in the parent strain. Each tested mutant was crossed with parent strain *pho1-44* and progenies of three full tetrads of each cross were tested for acid phosphatase activity and thiamine uptake. In all cases deregulated thiamine uptake rates observed for the mutants cosegregated in a 2:2 manner with deregulated acid phosphatase expression. This indicates that thiamine transport is under the control of genes *tnr1*, *tnr2*, *tnr3* and *thi1*.

Epistasis and dominance/recessiveness relationships of *tnr* and *thi1* mutations: To determine the epistatic relationships between *tnr* and *thi* mutations 12 different double mutants (*thi1-1tnr1-18*, *thi1-1tnr1-9*, *thi1-23tnr1-18*, *thi1-23tnr1-9*, *thi1-1tnr2-2*, *thi1-1tnr2-11*, *thi1-23tnr2-2*, *thi1-23tnr2-11*, *thi1-1tnr3-5*, *thi1-1tnr3-10*, *thi1-23tnr3-5*, *thi1-23tnr3-10*) were grown in the absence and presence of thiamine and examined for acid phosphatase activity. They revealed no or only residual growth on MM and acid phosphatase activity levels were as in *thi1* mutants. Alleles *thi1-1* and *thi1-23* are apparently epistatic over the examined *tnr* alleles. *Thi1tnr3* double mutants grow poorly in MM containing thiamine or in YE indicating that mutations in the *thi1* and *tnr3* gene are harmful for cells when present together.

Dominance/recessiveness relationships of *tnr* and *thi1* mutations were analyzed in diploids. The following mutant alleles were tested: *tnr-1*, *tnr1-9*, *tnr1-18*, *tnr2-2*, *tnr2-11*, *tnr3-5*, *tnr3-9*, *tnr3-19*, *thi1-1*, *thi1-7* and *thi1-23*. The homozygous diploids revealed the same acid phosphatase and growth phenotypes as the haploid mutants. All heterozygous diploids exhibited the same acid phosphatase activities as homozygous wild-type diploids. Heterozygous *thi1* diploids were prototrophic for thiamine. Mutations in the genes *tnr1*, *tnr2*, *tnr3* and *thi1* are clearly recessive.

DISCUSSION

We screened mutants defective in the regulation of thiamine repressible acid phosphatase and showed that these mutants do not properly regulate mRNA levels of genes *pho4* and *nmt1* in response to the thiamine signal. The mutants map in four genes which are apparently involved in thiamine-regulated gene expression. Genes *tnr1*, *tnr2* and *tnr3* act negatively, and gene *thi1* positively on the expression of the two examined thiamine regulated genes. All tested *tnr* and *thi1* alleles of the four genes are recessive and *thi1* mutations are epistatic over those of the *tnr* genes. We assume that the gene products of the regulatory genes are involved in thiamine-mediated transcription control. If this is true one would expect common sequence motifs in the promotor region of the *pho4* and *nmt1* gene. Indeed these exist. The longest is TATAAAACAAA which occurs at position −606 to −593 in *pho4* and −663 to −653 in *nmt1*. Whether this sequence or shorter ones not mentioned here are really involved in transcriptional control remains to be seen.

Genes *pho4* and *nmt1* are involved in dephosphorylation of thiamine phosphates and thiamine biosynthesis, respectively. Expression of other genes involved in thiamine metabolism may also be under the control of genes *tnr1*, *tnr2*, *tnr3* and *thi1*. Indeed, as reported in this communication, we also find that thiamine transport which is a thiamine-regulated process in wild-type cells is deregulated in *tnr* and *thi1* mutants.

Biosynthesis of thiamine occurs by the independent formation of the pyrimidine and thiazole moieties which are condensed in subsequent reactions to thiamine monophosphate. The biosynthetic pathways for the two moieties have not yet been elucidated, and nothing is known about their regulation (for review, see YOUNG 1986). Our results from *S. pombe* start to suggest that the thiamine biosynthetic pathway is under complex regulatory control. At least one step in the biosynthesis of the pyrimidine moiety (controlled by gene *nmt1*) is regulated. Our observations that *thi1*

mutants, being strongly repressed in the pyrimidine synthesis, do not grow in MM supplemented with pyrimidine, but do so when supplemented with thiazole also suggest that thiazole biosynthesis is regulated. All *tnr* mutants exhibit a similar phenotype concerning *pho4* and *nmt1* expression. *tnr3* mutants however differ from the other mutants and the parent strain by their unusual high intracellular thiamine levels. This could suggest that mutations in the *tnr1* and *tnr2* gene affect only expression of some regulatable genes involved in thiamine biosynthesis, whereas lesions in the *tnr3* gene derepress the whole biosynthetic pathway.

Little is known yet about the effect of thiamine on gene expression in other organisms. We showed that mRNA levels of the *pho3* gene of *S. cerevisiae* previously believed to code for a constitutively expressed acid phosphatase is in fact thiamine repressible (M. E. SCHWEINGRUBER *et al.* 1986). KAWASAKI *et al.* (1990) recently reported that the four enzymatic activities responsible for the condensation of the thiazole and pyrimidine moiety are high in *S. cerevisiae* cells grown in the absence of thiamine but low in cells grown in the presence of the vitamin. The activities are also low in a *pho6* mutant which exhibits repressed levels of thiamine-repressible acid phosphatase and thiamine auxotrophy. In view of our results it seems likely that regulation of these four enzymatic activities by thiamine and the *pho6* gene occurs at the transcriptional level. It will be interesting to see whether gene *thi1* from *S. pombe* corresponds to *pho6* from *S. cerevisiae*.

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Note added in proof: We recently found that expression of gene *thi2* which is responsible for thiazole synthesis (SCHWEINGRUBER *et al.* 1991) is also repressible by thiamine and under the control of the regulatory genes *tnr1*, *tnr2*, *tnr3* and *thi1*.

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