**nmt1** of Fission Yeast

**A HIGHLY TRANSCRIBED GENE COMPLETELY REPRESSED BY THIAMINE***

Kinsey Maundrell

*From the Sclavo Research Centre, Via Fiorentina 1, 53100 Siena, Italy*

The first fully repressible gene in fission yeast is described. In minimal medium it is highly transcribed producing a mRNA which is 50–100 times more abundant than the *cyc1* mRNA. By contrast, in minimal medium supplemented with thiamine at a concentration of 0.5 μM or greater the transcript is undetectable. The gene has been called *nmt1* (for no message in thiamine). The 5’ and 3’ ends of the transcript have been mapped and indicate an unspliced mRNA of 1.4 kilobases with no evidence of heterogeneity at either end. The single major open reading frame encodes a protein of 39 kDa. The gene product is most likely involved in thiamine biosynthesis and consistent with this is the observation that the *nmt1::ura4* disruption strain is a thiamine auxotroph. The kinetics of transcriptional repression and induction have been studied. Addition of thiamine to log phase cells growing minimal medium results in complete disappearance of the *nmt1* message within 3 h. Removal of thiamine from the medium produces the first detectable message after 10 h and maximal steady-state levels after 16 h. Nuclear “run on” experiments demonstrate that control is exerted at the level of transcription initiation. The *nmt1* promoter has been subcloned, and thiamine-mediated transcriptional control has been transferred to the bacterial reporter gene chloramphenicol acetyltransferase.

Transcription initiation is a major control point in the expression of eukaryotic genes. Loss of transcriptional control can have profound consequences for the cell including uncontrolled proliferation and oncogenic transformation. Not surprisingly this process has been the subject of intensive investigation in recent years.

The yeast *Saccharomyces cerevisiae* has proved to be a valuable experimental model. In general, the organization of yeast promoters is similar to those of higher eukaryotes. A TATA box is usually found upstream of the mRNA initiation site albeit at a variable distance of between 40 and 120 base pairs, and upstream regulatory elements, analogous to eukaryotic enhancers, have been shown to be the specific targets for positive and negative regulatory factors (for a review see Guarente, 1987). The availability of a variety of inducible genes, and the ease with which the genetic and molecular genetic approaches can be applied in yeast has led to the identification of *cis* - and *trans*-acting elements in a number of instances.

Particularly revealing has been the analysis of genes involved in amino acid biosynthesis which are co-ordinately controlled in response to amino acid starvation (Delforge et al., 1975). The upstream region of each of these genes contains a 9-base pair palindromic sequence 5′-ATGAC(G/C)TCAT-3′ (Donahue et al., 1983) which subsequent analysis has shown to be a target for the positive *trans*-activating protein GCN4 (Hill et al., 1986). The GCN4 protein contains two functionally distinct domains, the C-terminal region involved in DNA binding and an internal acidic region which activates the transcriptional machinery (Hope and Struhl, 1986). Curiously, the DNA-binding domain shows unexpectedly high amino acid sequence homology with the DNA-binding domain of the *jun* oncprotein (Vogt et al., 1987), a component of the transcription activation complex of HeLa cells, AP-1. Moreover, AP-1 recognizes a 9-base pair target sequence almost identical to the sequence recognised by GCN4 (Angel et al., 1987; Lee et al., 1987), and in an appropriate protein chimera the *jun* protein is capable of regulating the transcriptional activity of the amino acid biosynthetic genes in yeast (Struhl, 1987). The human oncogene, *fos*, is another transcriptional activator in human cells which binds DNA by associating with AP-1 complex (Chiu et al., 1988). *fos* is also capable of stimulating transcription of yeast genes when linked to an appropriate DNA-binding motif (Lech et al., 1988). From these data it seems probable that much of the basic machinery of transcriptional control has been highly conserved throughout eukaryotic evolution.

In *Schizosaccharomyces pombe*, similarity with the transcriptional machinery of higher eukaryotes is if anything even more pronounced. The TATA element is located a more uniform 25–30 base pairs upstream of the start of transcription (Russell, 1983) exactly as in mammalian cells (Benoist et al., 1986). The upstream region of each of these genes contains a g-base pair palindromic sequence Sd-ATGA(C/G)TCAT-3′ (Donahue et al., 1983) which subsequent analysis has shown to be a target for the *trans*-activating protein GCN4 (Angèl et al., 1987). S. pombe also has an AP-1-like factor which gives an identical footprint on SV40 DNA to human AP-1 (Jones et al., 1988). Up to now, however, a more detailed analysis of transcriptional control in fission yeast has not been possible because of a lack of known transcriptionally regulaatable genes in this organism. Thus far only *pho1* and *pho4* which code for two acid phosphatases have been isolated, however, neither has an abundant transcript and neither is completely repressed in the uninduced state (Maundrell et al., 1985a; Seveingruber et al., 1986). In this present study I report the isolation of *nmt1*, the first fully regulatable gene in *S. pombe*.
It is among the very highly expressed genes in this organism and is totally repressed in medium containing thiamine.

**Materials and Methods**

**Strains**—All S. pombe strains used were derived from the wild-type 972h and are described in the text. Bacterial strains used were JM101TR (supE, thi, rpsL-15, F-, lac-proAB, lacZAM15) for routine work, and JA221 (recA1, leuB6, trpD4E5, hisD2, lacY, C900) when selecting for recombinants containing the S. cerevisiae LEU2 gene which complements the leu8 mutation of Escherichia coli. Processes and General Techniques—Yeast media and transformation were as described by Beach and Nurse (1981). For bacteria, standard media were used throughout. CAT² activity was assessed as Jones et al. (1988). General procedures used in cloning and blotting are described in Maniatis et al. (1982).

**Preparation of RNA and Northern Blotting**—Total RNA was prepared as described by Maundrell et al. (1985a) and resuspended in TE at 2 μg/ml. For Northern blotting, 10 μg of RNA was denatured in 0.5 M glyoxal and 50% Me2SO and run in 1.2% agarose containing 25 mM phosphate buffer, pH 6.5. RNA was transferred to Biodyne B in 20 × SSC (1 × SSC contains 0.15 M NaCl and 0.15 M sodium citrate) by capillary blotting overnight. The membrane was prehybridized in 50% formamide, 5% Denhardt’s (1 × Denhardt’s contains 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyethylene glycol), 1 M NaCl, 0.1% SDS, 5 μg EDTA, 100 μg/ml calf thymus DNA at 42°C for 2 h and hybridized overnight in the same solution containing 32P-labeled probe made by random priming gel-purified restriction fragments (Feinberg and Vogelstein, 1984) as described in the legends to figures.

**cDNA Probes and Differential Screening of the Genomic Library**—Wild-type S. pombe 972h was grown to mid log phase (8 × 10⁶ cells/ml) in 200 ml of minimal medium or 200 ml of minimal medium containing 2 μM thiamine and RNA was prepared. Polyadenylated mRNA from each preparation was selected on oligo(dT) cellulose (Maniatis et al. 1982) and used as template for synthesis of 32P-labeled cDNA (Guthrie and Hoffmann, 1984). A genomic library made from a partial HindIII digest of S. pombe DNA cloned into DB262 (Wright et al., 1986) was plated and transferred to nitrocellulose filters. The filters were incubated at 65°C overnight with 10⁶ cpm/ml cDNA (+ thiamine) in 6 × SSC, 2 × Denhardt’s, 0.5% SDS, 100 μg/ml herring sperm DNA. After hybridization the filters were washed in 2 × SSC, 0.5% SDS, 50°C (1 wash, 30 min) and exposed to autoradiographic film. The first probe was then removed in 50% formamide, 1 × SSC at 70°C and the filters reprobed with cDNA (− thiamine) as above.

From the results of differential hybridization, several colonies were selected and one of these contained nmt1, the subject of the present report. To obtain the flanking sequence upstream of nmt1, the same genebank was hybridized to the 32P-labeled nmt1 probe and a 4.1-kb fragment was recovered which contains the original HindIII fragment of 2.4 kb and a second HindIII fragment of 1.7 kb which was shown by Southern blotting to be contiguous with and upstream of the 2.4-kb fragment in the genome and not the result of oligonucleotide cloning.

**DNA Sequencing and in Vitro Mutagenesis**—DNA fragments were cloned into either M13mp19 or pUC119 and sequenced in both directions from deletions made using Exonuclease III and S1 nuclease (Henikoff, 1984). Mutagenesis was performed using the UWGGC software (Devereux et al., 1984). Site-directed mutagenesis was performed as described (Maundrell et al., 1988) and in more detail elsewhere.²

²S′ and 3′ Mapping of the nmt1 Transcript—The 5′ end of nmt1 mRNA was mapped by two methods: (a) by primer extension using a synthetic 18-mer 5′-CCTCACTCACTATCCTCCTGCTTCA-3′ complementary to the coding sequence between positions +90 and +109. The oligonucleotide was 32P-labeled with polynucleotide kinase and annealed and extended with reverse transcriptase as described (Grimm et al., 1988); and (b) by S1 mapping using a uniformly labeled probe. In this case the probe was made by annealing the oligonucleotide above to single strand M13 DNA carrying the nmt1 coding strand, extending the primer using the “Klenow” enzyme in the presence of 32P-labeled dCTP and digesting with HindIII. The reaction mixture was denatured and radioactive 293 nucleotide fragment was purified on a preparative sequencing gel and used for S1 mapping essentially as described (Berk and Sharp, 1977).

The 3′ end of the mRNA was mapped by S1 mapping. The probe in this case was made from an Exonuclease III-derived mutant, carrying a 3′ deletion which extends into the clone as far as position +1310. Single strand M13 phage DNA containing this deletion was annealed with the 17-mer universal primer, extended with Klenow enzyme, and digested with NcoI. The 371-nucleotide fragment was purified and used for S1 mapping as above.

**Transcriptional “Run On”**—The procedure was based on that described by Elion and Warner (1986). Wild-type cells were grown to mid log phase in minimal medium supplemented with 2 μM thiamine. From each culture, 3 × 10⁸ cells were harvested by centrifugation, washed in TMN (10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl) and resuspended in 950 μl of H₂O. 50 μl of 10% sodium lauryl sarcosine was added and the suspension incubated on ice for 30 min. Cells were pelleted for 2 min in an Eppendorf centrifuge, and the pellet was resuspended to remove all detergent. The final pellet was resuspended in 100 μl of reaction mixture (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 1 mM MnCl₂, 0.5 mM dithiothreitol, 0.5 mM ATP, 0.25 mM GTP, 0.25 mM CTP, 10 mM phosphocreatine, 10 μg/ml phosphocreatine kinase) containing 10 μl (100 μCi) of [α-32P]UTP and incubated at 25°C. After 5 min 1 ml of ice-cold TMN containing 1 mM UTP was added. Cells were pelleted, and RNA was recovered as described (Maundrell et al., 1985a). Identical slot blots containing 5 μg/slot of various immobilized plasmid DNAs (see Fig. 3) were hybridized overnight to 5 × 10⁶ cpm of each RNA probe using the same hybridization conditions employed for Northern blotting. Washing conditions were the same as described above for differential screening of the genomic library and filters were exposed to Kodak X-onomatAR for 2 days.

**Disruption of nmt1**—The procedure was essentially that described by Grimm et al. (1988). The 2.4-kb HindIII fragment containing the nmt1 coding region was cloned into pUC19 and the 0.8-kb Xhol-NcoI fragment was replaced by a 1.8-kb segment containing the ura4+ gene. The plasmid was digested with PstI which cuts on either side of the polylinker and approximately 10 μg of the linear fragment containing the disrupted gene was isolated by preparative electrophoresis and used to transform the diploid strain h/h ade6-704/ade6-704 leu1-32/leu1-32 ura4-d18/ura4-d18 to uracil prototrophy. Of 10 stable Ura+ transformants analyzed by Southern blotting, two were the result of replacing one nmt1 allele of the diploid with a single copy of the disrupted allele. Sporulating h/r² revertants were obtained from both homologous integrants and tetrads were analyzed by microdissection (Gutz et al., 1974). Identical results were obtained with both strains.

**Results**

**Isolation of Thiamine Regulatable Genes by Differential Hybridization**—As part of a search for inducible genes in fission yeast, I have screened a genomic library for sequences whose expression is sensitive to the presence of exogenous thiamine. In S. pombe, thiamine is not an essential requirement for growth although it is maintained at high intracellular concentration which suggests that as in S. cerevisiae, active biosynthesis of the vitamin can occur. The weakly thiamine-repressible gene pho4 has been previously described (Schweinberger et al., 1986). For a more extensive search for thiamine-regulated genes, cells were grown in either minimal medium or minimal medium supplemented with 2 μM thiamine, and the poly(A)+ mRNA was prepared from each culture and used to prime the synthesis of corresponding 32P-labeled cDNA probes. A genomic library consisting of partially digested HindIII fragments cloned into pDB269 (Wright et al., 1986) was then screened sequentially with each of the two cDNA probes. Several different clones were isolated all of which hybridized more strongly to cDNA derived from cells grown in minimal medium alone. One of these clones contained a single 2.4 kb HindIII fragment, and hybridized as strongly as any other clone in the gene bank, suggesting that in the
induced state it produces a highly represented cellular mRNA. The gene encoded by this fragment was subsequently referred to as nmt1 (see next section) and is the subject of the present report.

Thiamine Is a Potent Inhibitor of nmt1 Expression—Northern blotting analysis of total S. pombe RNA using the 2.4-kb HindIII fragment as probe revealed a single transcript with an estimated molecular weight of approximately 1.3 kb (see legend to Fig. 1). Expression of this transcript was highly sensitive to the presence of thiamine. As shown in Fig. 1, thiamine concentrations in excess of 0.05 μM resulted in a progressive decrease in the steady-state level of the nmt1 mRNA while at a concentration of 0.5 μM thiamine or greater, no hybridization at all could be detected even after long exposures of the blot. For this reason the gene was designated nmt1. As a control the same RNA samples were run in parallel and probed to reveal the cycl mRNA (Fig. 1, lower panel). The probe in this case was the 0.54-kb EcoRI-SalI fragment from pPoCYC(0.54) (Russell and Hall, 1982). The probes for both blots were labeled to the same specific activity, and the same number of cpm were used for each hybridization (see legend to Fig. 1). The results using the cycl probe confirm that approximately equal amounts of RNA were loaded in each lane. Moreover, quantitation based on densitometric scanning (see legend to Fig. 1) indicates that nmt1 mRNA when fully expressed is 50–100 times more abundant than cycl mRNA.

To study the kinetics of thiamine repression, a cell culture was grown to early log phase in minimal medium and supplemented with 2 μM thiamine at T0. Samples were taken at 1-h intervals thereafter for RNA preparation. A Northern blot probed to reveal the nmt1 mRNA is shown in Fig. 2A. The nmt1 message completely disappears from the cell within 3 h or about one cell cycle under these conditions.

Thiamine Acts as a Repressor of Transcription Initiation—Disappearance of mRNA from the cell as determined by Northern blotting can be due either to inhibition of transcription initiation or to an increase in the rate of mRNA turnover. To investigate this in the case of nmt1, transcriptional run on experiments were performed. Cells were harvested in mid-log phase after growth in minimal medium or minimal medium containing 2 μM thiamine, and cells from each culture were permeabilized and incubated with [α-32P]UTP for 5 min essentially as described by Elion and Warner, (1986; see “Materials and Methods”). Radiolabeled RNAs were then extracted and hybridized to identical slot blots carrying various immobilized plasmid DNAs. As can be seen from the results shown in Fig. 3 the level of hybridization of radioactive RNAs to the two control genes ure4 and cycl is independent of the presence of thiamine in the growth medium, while the pUC119 vector alone does not hybridize to any cellular message after the high stringency washing conditions employed. On the other hand, the nmt1 containing plasmid reacts very strongly with RNA from cells grown in minimal medium alone and hardly at all with RNA from cells grown in the presence of thiamine. These results indicate that the main effect of

FIG. 2. A, Northern blot analysis showing the kinetics of repression of nmt1 transcription by thiamine. 972h− cells were grown to early log phase (2 × 10⁶ cells/ml) in minimal medium. At T0, 10⁶ cells were withdrawn for RNA preparation, and the remainder of the culture was supplemented with 2 μM thiamine. Further aliquots of 10⁶ cells were taken at intervals as indicated. RNA was extracted and analyzed as described under “Materials and Methods.” B, Northern blot analysis showing the kinetics of induction of nmt1 expression. 972h− cells were grown to mid-log phase in minimal medium containing 2 μM thiamine and an aliquot removed for RNA preparation (T0). The cells were washed in minimal medium and resuspended at 10⁶ cells/ml in fresh minimal medium. Further aliquots were withdrawn at intervals as indicated. After 10 h, as cells approached the end of log phase they were diluted in fresh prewarmed minimal medium for the analysis of later time points. RNA was extracted and analyzed as described under “Materials and Methods.”
exogenous thiamine on nmtl expression is to drastically re-

duce the rate of transcription initiation.

Structure of the nmtl Gene—The 2.4-kb HindIII fragment

containing the nmtl gene was completely sequenced in both
directions from a series of deletion mutants prepared by
digestion with Exonuclease III and nuclease SI (Henikoff,
1984). A major unsliced open reading frame of 346 amino
acids was found which potentially encodes a 39-kDa polypep-
tide (Fig. 4). A search of the EMBL and Genbank databases
showed no obvious homologies with other known proteins.

In order to analyze the 5′ regulatory region of nmtl, a more
extensive upstream region was obtained by reproping the same
partial HindIII gene bank with the radiolabeled 2.4-kb insert.
Among the positive colonies, one was found which contained
the original 2.4-kb fragment together with an additional frag-
ment of 1.7 kb. Southern blots of total S. pombe genomic
DNA digested with a variety of diagnostic enzymes and probed
with each of the two fragments separately, showed that the
1.7-kb fragment was contiguous with and upstream of the 2.4-
kb fragment (data not shown). Most of the 1.7-kb fragment
was also sequenced in both directions. The nucleotide se-
quence and restriction map of the nmtl locus is shown in Fig.
4.

The 5′ end of nmtl mRNA was determined by primer extension using an end-labeled oligonucleotide (Fig. 5A) and
by SI mapping using a uniformly labeled probe (see “Materials
and Methods”). Both sets of data revealed that transcription
initiates on the A residue at position -69 and confirm that
no splicing occurs in this region. The sequence TATATAAA
occurs from -34 to -27 relative to the start of transcription.
The 3′ end of the transcript was determined by SI mapping
using a continuously labeled probe of 371 nucleotides extend-
ing downstream from the NcoI site within the coding region
(see “Materials and Methods”). A single protected fragment
of 244 nucleotides (Fig. 5B) locates the 3′ end of the message
142 nucleotides after the stop codon. The predicted length of
the mRNA based on mapping of the ends is therefore 1252
nucleotides, close to the value determined by Northern blot-
ing. From these data I conclude that transcription of the
nmtl gene produces a simple unspliced mRNA and that there
is no detectable heterogeneity at either end (see Fig. 4B).

The Cloned Fragment Contains the Transcriptional Regu-

latory Elements—To ascertain whether the 4.1-kb cloned
fragment was capable of regulated transcription of nmtl, the
complete region in the original vector DB262, was trans-
fomed into fission yeast and grown in the presence or absence
of thiamine. RNA was prepared and analyzed by Northern blotting (Fig. 6). Densitometric scanning of the blot shown in
Fig. 6 shows that cells grown in inducing conditions (lane c)
contain approximately twice as much nmtl mRNA as control
cells transformed with DB262 alone (lane a). In repressing
conditions nmtl mRNA is undetectable both in the control
(lane b) and in the nmtl transformant (lane d).

To be certain that the increased transcript level comes from
the plasmid, a derivative of the original clone was made in
which the 185-base pair BamHI-BamHI fragment was deleted
from the nmtl coding region (see Fig. 4). Analysis of the RNA
from cells transformed with the deletion clone is shown in
Fig. 6 (lanes e and f). As expected, two RNAs are detected, a
full-length chromosomal transcript and a shorter transcript
derived from the plasmid. Estimates based on densitometric
scanning show that in inducing conditions (lane e), slightly
more than half of the message (55%) is transcribed from the
plasmid. When cells are grown in thiamine, transcription from
both the chromosome and the plasmid is totally repressed.

From these data I conclude that the cloned nmtl gene and
its flanking regions include all the regulatory elements nec-

essary for thiamine-mediated control of transcription, and
second, that normal regulation can occur even in the presence
of multiple copies of the promoter.

A nmtl Disruption Strain Is a Thiamine Auxotroph—As a
means of investigating the function of nmtl, the chromosomal
gene was disrupted essentially as described by Grimm et al.
(1988). The region between the Xhol and NcoI sites of the
coding sequence was replaced with the ura4 gene and the
linear fragment was isolated and used to transform the diploid
strain h+/h-, ura4-d18/ura4-d18, leu1-32/leu1-32, ade6-704/ade6-704 to uracil prototrophy. Restriction analysis of 10
stable Ura+ diploid transformants showed two which had the
predicted pattern for homologous integration at the nmtl
 locus (data not shown). A HindIII digest of such a diploid
(Fig. 7, lane D) produced equimolar amounts of a 2.4 and a
3.4-kb band corresponding to the wild type and disrupted
fragments, respectively. A sporulating h+/h- diploid revertant
was selected by exposure to iodine vapor and the products of
meiosis analyzed by tetrads. In all cases the four spores were
viable on rich medium. DNA from each of the spores was
analyzed by Southern blotting and as expected showed two wild-type and two disrupted alleles, the two
disrupted alleles segregating with the Ura+ phenotype (Fig.
7A). The growth characteristics of each of the four spores
from a single tetrad cultured in minimal medium (supple-
mented with adenine, leucine, and uracil) with or without
exogenous thiamine is shown in Fig. 7B and Table I. The
results show that for nmtl cells (upper panel) addition of
thiamine to the culture medium produces a stimulation in the
rate of cell doubling of approximately 10%, showing indeed
that in minimal medium the availability of thiamine is rate
limiting for cell growth. Cells carrying the nmtl::ura4 dis-
rupted allele (lower panel) also grow in minimal medium
containing thiamine and show the same accelerated pattern
of growth shown by wild-type cells. However, in the absence
of exogenous thiamine, cells which lack a functional nmtl
allele are not capable of sustained growth.

Thiamine-mediated Control of Transcription Can Be Trans-
ferred to Heterologous Genes—As a first step toward identi-
fying the elements responsible for control of nmtl transcrip-
tion, the flanking sequences upstream and downstream of the
nmtl coding region were isolated and used to construct the
expression vector pREP1 shown in Fig. 8. For this construct
the 1.2-kb fragment which extends from the upstream Bcll
site to the initiator ATG was judged sufficient to contain the
complete promoter. To facilitate fusion of reporter or other
heterologous genes, the sequence around the initiator codon
was modified by in vitro mutagenesis to incorporate recog-
FIG. 4. A, restriction map of the *nmt1* gene and its flanking sequences. The solid box represents the putative *nmt1* coding region, the stippled area represents the extent of the sequence determined, the boxed area contains within HindIII sites represent limits of the 4.1-kb cloned sequence. The sequencing strategy is shown by the arrows below the stipled area. Arrows starting with solid circles indicate sequences primed by specific oligonucleotides. B, nucleotide sequence of the genomic region containing *nmt1*. The region shown was sequenced in both directions with the combination of exonuclease digests (Henikoff, 1984) and custom-made oligonucleotide sequencing primers (see A above). A single major open reading frame which codes for the putative *nmt1* gene product is shown. The horizontal arrow indicates the site of transcription initiation as determined by S1 mapping and primer extension. This site occurs 27 base pairs downstream of the sequence TATAAA (boxed). The two underlined sequences are direct repeats also found in the promoter of the thiamine-sensitive PH03 gene of *S. cerevisiae*. The vertical arrow following the open reading frame indicates the site of transcription termination as indicated by S1 mapping. Transcript mapping data are given in Fig. 5.
between the PstI and StuI sites of a S. pombe shuttle vector containing the LEU2 gene of S. cerevisiae and the ars1 of S. pombe cloned into the HindIII and EcoRI sites, respectively, of the pUC119 polylinker (see Hindley et al., 1987). This construct was designated pREP1. An integrating plasmid, pRIP1, was derived from pREP1 by deleting the PstI and SstI sites of a shuttle vector. A more detailed description of this and other vectors based on the nmt1 control elements will be described elsewhere.2

To test the activity of the nmt1 promoter, the bacterial gene chloramphenicol acetyltransferase was used as a convenient reporter gene (Gorman et al, 1982). An NdEl site was introduced at the initiator methionine codon by in vitro mutagenesis and the 0.8-kb NdEl-HindIII containing the entire coding region was subcloned between the nmt1 promoter and transcriptional stop elements of pREP1 and pRIP1. S. pombe transformants were obtained which carried either multiple extrachromosomal copies of pREP1-CAT or a single

Table I

| Strain | Genotype | Doubling time |
|--------|----------|---------------|
|        | +Thiamine | -Thiamine     |
| Spore 1 | nmt1     | 2.3           | 2.5          |
| Spore 2 | nmt1::ura4 | 2.2           |              |
| Spore 3 | nmt1     | 2.3           | 2.8          |
| Spore 4 | nmt1::ura4 | 2.2           |              |
|         | 972h      | 1.9           | 2.1          |

* All spores are ura4-d18, leu1-32, ade6-704.

* Data for spores is derived from Fig. 7B.

FIG. 5. Mapping of the 5' and 3' termini of the nmt1 transcript. A, primer extension to localize the 5' extremity of the nmt1 transcript. A synthetic oligonucleotide complementary to the nmt1 mRNA between positions +90 to +109 was annealed to mRNA from cells grown in minimal medium or minimal medium containing 2 μM thiamine and extended toward the 5' end of the mRNA using reverse transcriptase in the presence of [α-32P]dCTP (see "Materials and Methods"). The products of the reaction were denatured and electrophoresed in parallel with the nmt1 gene sequenced from the same oligonucleotide primer. Lanes 1-4 show the G, A, T and C reactions of the nmt1 sequence; lane 5 shows primer extension results using RNA obtained from cells grown in minimal medium; lane 6 shows primer extension results using RNA obtained from cells grown in minimal medium containing 2 μM thiamine. Lanes 5' and 6' are longer exposures of lanes 5 and 6. B, S1 nuclease protection analysis to localize the 3' end of the nmt1 transcript. A 371 nucleotide probe complementary to, and extending beyond, the 3' end of the nmt1 mRNA was prepared and used for S1 mapping as described under "Materials and Methods." Lane 1 shows the purified probe; lane 2 shows the probe reacted without RNA and without S1 nuclease; lane 3 shows the probe alone digested with S1 nuclease; lane 4 shows the probe reacted with RNA from cells grown in minimal medium then digested with S1 nuclease; lanes 5-9 show the C, T, A, and G reactions of M13mp18 sequenced using the universal 17-mer primer.

FIG. 6. Northern blot analysis showing the expression of nmt1 on a high copy number plasmid. leu1−32 cells were transformed with DB262 alone (lanes a and b), DB262 containing the 4.1-kb nmt1 gene (lanes c and d), and DB262 containing the 4.1-kb nmt1 gene from which the 186-base pair BamHI-BamHI fragment was deleted from the coding region (lanes e and f). Cells were grown in minimal medium in the absence (lanes a, c, e) or presence (lanes b, d, f) of thiamine. RNA was extracted and analysed as described under "Materials and Methods.

FIG. 7. A, Southern blot analysis of the nmt1::ura4 gene disruption strain. One copy of nmt1 in the diploid strain *h*/*h* ade6−704/ade6−704 leu1−32/leu1−32 ura4-d18/ura4-d18 was disrupted by replacing the 0.8-kb XhoI-NcoI fragment with the 1.8-kb S. pombe ura4 gene (see "Materials and Methods"). DNA from the diploid digested with HindIII is shown (D). The two hybridizing fragments of 2.4 and 3.4 kb correspond to the wild-type and disrupted genes, respectively. The diploid was sporulated and DNA isolated from each of the four spores (Maundrell et al., 1985). The wild-type (spores 1 and 3) and disrupted (spores 2 and 4) genes segregate with the Ura− (−) and Ura+ (+) phenotypes, respectively. B, growth curves showing the thiamine dependence of the nmt1::ura4 disruption strain. Each of the four spores in (A) above was cultured in minimal medium supplemented with adenine leucine and uracil either with (solid symbols) or without (open symbols) thiamine. Cells were counted at intervals using a haemocytometer. Upper panel shows nmt1 cells derived from spore 1 (diamonds) and spore 3 (triangles). Lower panel shows nmt1::ura4 cells derived from spores 2 (squares) and 4 (circles).
Fig. 8. Construction of the expression vector pREP1. A, schematic representation of the 4.1-kb genomic fragment containing the nmt1 gene. The area shown in black is the nmt1 coding region. The extent of the nmt1 transcript is indicated by the arrow. The stipled areas represent the regions used for vector construction. B, structure of pREP1. The nmt1 promoter was modified in two ways: first the internal NdeI site at position -768 was destroyed as shown; second the sequence around the ATG was altered by site-directed mutagenesis to incorporate restriction sites for NdeI and SalI. The transcriptional stop element extending from the nmt1 stop codon TAA to a site approximately 1 kb downstream was amplified by polymerase chain reaction and BamHI, SmaI, and SstI sites added as indicated. Both elements were assembled into the polylinker of pUC119 which contained in addition the LEU2 gene of S. cerevisiae and ars1 of S. pombe cloned into the HindIII and EcoRI sites, respectively. The resulting vector was termed pREP1. An integrating version of this vector, called pRIP1, was obtained by deleting ars1.

Fig. 9. CAT activity in extracts of S. pombe transformants. leu1-32 transformants containing either pREP1-CAT (lanes a and b) and pRIP1-CAT (lanes c and d) were grown in minimal medium (lanes a and c) or minimal medium containing 2 μM thiamine (lanes b and d). 10⁶ cells from each culture were processed for CAT assay as described by Jones et al. (1988).

integrated copy of pRIP1-CAT. Parallel cultures of each transformant were grown in minimal medium either with or without thiamine and CAT activities in the respective extracts were assayed (Fig. 9). A transformant containing pREP1-CAT is shown in lanes a and b; a transformant containing pRIP1-CAT is shown in lanes c and d. In both cases addition of thiamine results in dramatic reduction of CAT expression. Estimates based on extract dilution indicate that CAT activity in the pREP1-CAT transformant is reduced approximately 200-fold in the presence of thiamine while CAT activity in the pRIP1-CAT integrant strain is reduced approximately 500-fold.

A systematic analysis of the thiamine responsive elements in the nmt1 promoter is in progress.

DISCUSSION

In this report I describe the first fully repressible gene to be isolated from fission yeast. In minimal medium, it is among the very highly transcribed genes in the cell; in minimal medium containing thiamine at a concentration of 0.5 μM or greater, gene expression is repressed and to judge from long overexposure of the Northern blot shown in Fig. 1, the transcript completely disappears from the cell. This gene has been designated nmt1. The kinetics of induction and repression are shown in Fig. 2. Addition of thiamine to cells growing in minimal medium results in complete disappearance of nmt1 mRNA within less than 1 cell cycle (Fig. 2A) indicating a rapid transcriptional response and also a high rate of mRNA turnover. In the reciprocal experiment in which thiamine is washed out from the medium and the cells shifted to minimal medium alone, induction of nmt1 transcription occurs after about 10 h in culture and maximal mRNA levels are obtained by 16 h. Transcriptional run on experiments show that thiamine control operates by inhibiting transcription initiation.

The cloned portion of the genome which spans nmt1 is 4.1 kb and contains all the sequences required for the thiamine mediated control of expression. This conclusion is based on the results in Fig. 6 which demonstrate that nmt1 on a high copy number plasmid is repressed in the presence of thiamine as completely as the endogenous chromosomal gene. Scanning the nmt1 promotor for repetitive elements, which in other eukaryotic genes have often been identified as targets for trans-acting molecules, revealed the presence of the 9-mer 5'-AAATAATAC-3' present at position -684 to -676 and an exact repeat at position -577 to -569 (underlined in Fig. 4B).

Experiments are in progress to test the functional significance of this sequence. The identical 9-mer also occurs at position -122 to -114 in the promotor of the only other thiamine sensitive gene sequenced to date, the PH03 gene of S. cerevisiae (Bawja et al., 1984). As discussed in the Introduction, cis-acting regulatory elements are often highly conserved among eukaryotes, even between yeast and man.

The function of the nmt1 gene product is not known and a search of the EMBL and Genbank databases showed no obvious homologies with other known proteins. From the pattern of expression, a likely function would be an involvement in thiamine biosynthesis, and support for this prediction comes from the finding that the nmt1::ura4 disruption strain is a thiamine auxotroph. Furthermore, overexpression of nmt1 results in approximately 10% stimulation of growth rate, similar to that obtained by adding thiamine to minimal me-
nmt1 of Fission Yeast

dium (Table I). The effect in both cases is probably to increase the concentration of intracellular thiamine and this further strengthens the notion that nmt1 is involved in thiamine production.

Why a gene involved in thiamine biosynthesis should be so highly expressed is not immediately clear. However, the observed stimulation of growth in the presence of exogenous thiamine indicates that the availability of the vitamin is rate limiting in minimal medium and therefore that selection has favored high level expression of the thiamine biosynthetic genes. The finding that overexpression of nmt1 also increases growth rate in minimal medium suggests that the nmt1 gene product performs a rate-limiting step in this pathway.

Further analysis of this system should enable a detailed investigation of transcription activation in fission yeast analogous to studies previously performed in budding yeast. In this paper I describe the construction of oligonucleotides, to A. Ruspetti and L. Fini for plates and media, to G. Corsi for artwork, and to C. Mallia for patient secretarial assistance.

Acknowledgments—I would like to express sincere thanks to Drs. M. Tommasino, J. Telford, and M. Melli for innumerable fruitful discussions during the course of this work and to all members of the Sclavo Research Centre for their continuous support and interest. In particular, thanks are due to Dr. G. Ratti and S. Ricci for synthesis of oligonucleotides, to A. Ruspetti and L. Fini for plates and media, to G. Corsi for artwork, and to C. Mallia for patient secretarial assistance.

REFERENCES

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729-739

Rajwe, W., Meyhack, B., Rudolph, H., Schweingruber, A.-M., and Hinnen, A. (1984) Nucleic Acids Res. 12, 7721-7739

Beach, D., and Nurse, P. (1981) Nature 299, 140-142

Derk, A. J., and Sharp, P. A. (1977) Cell 12, 721-732

Benois, C., O'Hare, K., Breathnach, R., and Chambon, P. (1980) Nucleic Acids Res. 8, 127-142

Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) Cell 54, 541-552

Delforge, J., Messenguy, F., and Wiame, J. (1975) Eur. J. Biochem. 57, 231-239

Devereux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 887-895

Donahue, T. F., Daves, R. S., Lucchini, G., and Fink, G. R. (1983) Cell 32, 89-98

Elion, E., and Warner, J. (1986) Mol. Cell. Biol. 6, 2089-2097

Feinberg, A. P., and Vogelstein, B. (1984) Anal. Biochem. 137, 266-267

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044-1051

Grimm, C. Kohli, J., Murray, J., and Maundrell, K. (1988) Mol. Gen. Genet. 215, 81-86

Guarente, L. (1987) Annu. Rev. Genet. 21, 425-452

Hill, D. E., Hope, I. A., Macke, J. P., and Struhl, K. (1986) Science 234, 451-457

Hindley, J., and Phear, G. A. (1984) Gene (Amst.) 25, 263-269

Hutz, H., Heiblot, H., Leupold, U., and Loprieno, N. (1974) in Handbook of Genetics (King, R. C., ed) pp. 1395-1446, Plenum Publishing Co., New York

Hinnen, A. (1984) Gene (Amst.) 28, 351-359

Langford, C. J., and Gallwitz, D. (1983) Cell 33, 459-472

Lee, W., Mitchell, P., and Tjian, R. (1987) Cell 49, 741-752

Maniatis, T., Frisch, E., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Maundrell, K., Nurse, P., Shonholzer, F., and Schweingruber, M. E. (1985a) Gene (Amst.) 30, 225-230

Maundrell, K., Wright, A. P. H., Piper, M., and Shall, S. (1985b) Nature 318, 571-572

Maundrell, K., Huchison, A., and Shall, S. (1985c) Nucleic Acids Res. 13, 5711-5722

Mount, S. M. (1982) Nucleic Acids Res. 10, 459-472

Russell, P. (1985) Nature 318, 78-80

Telford, J. E., and Doolittle, R. F. (1987) J. Biol. Chem. 262, 8581-8586

Vogt, P. K., Bos, T. J., and Doolittle, R. F. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3316-3319

Wright, A. P. H., Maundrell, K., Heyer, W.-D., Beach, D., and Nurse, P. (1986) Plasmid 15, 156-158
nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine.
K Maundrell

J. Biol. Chem. 1990, 265:10857-10864.

Access the most updated version of this article at http://www.jbc.org/content/265/19/10857

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/19/10857.full.html#ref-list-1