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# Transcriptional Regulation of the Cell Cycle-Dependent Thymidylate Synthase Gene of *Saccharomyces cerevisiae*

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**We have previously shown that transcript levels expressed from the yeast *TMP1* gene fluctuate periodically during the yeast cell cycle. However, it was not known whether periodic expression resulted from a regulatory mechanism acting at the level of transcription or a regulatory mechanism acting at the level of cell cycle stage-dependent changes in the stability of the *TMP1* transcript. In this report we now show that the periodic expression of *TMP1* transcript is primarily controlled at the level of its transcription by sequences which are upstream of its transcription initiation sites. We also localized the upstream sequences necessary for periodic transcription to a 150-base-pair region and show that this region encodes an element(s) with the properties of a periodic upstream activating sequence. The regulatory region defined in this study apparently does not contain consensus sequences similar to those reported for the cell cycle-regulated *HO* endonuclease or for the histone *H2A* and *H2B* genes of *Saccharomyces cerevisiae*.**

One approach to investigating the processes which control eucaryotic cell division is to identify and study those genes which are differentially regulated during the cell cycle. If progression through the cell division cycle results from the sequential transient expression of dormant genes, then identifying the factors which govern the expression of these genes is of considerable interest. In the yeast *Saccharomyces cerevisiae*, only a few genes are currently known to exhibit differential expression during the cell cycle. These include the thymidylate synthase gene *TMP1* (25), the histone *H2A* and *H2B* genes of the *TRT1* locus (12), the *HO* endonuclease gene (20), the thymidylate kinase gene *CDC8* (28), and the DNA ligase gene *CDC9* (23).

Previous studies have shown that the regulation of the periodically expressed yeast histone genes results from complex controls acting at both the transcriptional and posttranscriptional levels (14, 21, 22). In addition, their proper expression may also involve the placement of these genes adjacent to an origin of DNA replication (21). Similarly, expression of the *HO* gene appears to be governed by complex controls acting transcriptionally and posttranscriptionally (4, 20). Nasmyth (20) has identified a 12-base-pair (bp) consensus sequence which is repeated many times within the URS2 region of *HO* and has been shown to be critically involved in the periodic expression of this gene.

We found that *TMP1* mRNA is transiently expressed during the cell cycle, with peak amounts occurring during late G1 and early S phase just after the *cdc28*/α-factor arrest point (25). In a recent study, White et al. (28) demonstrated that the periodic expression of *TMP1*, *CDC8*, and *CDC9* mRNA was coincident, while the histone *H2A* gene was expressed distinctly later in the cell cycle. This was shown by analyzing the times at which these genes were expressed after release from G1 arrest and also by showing that only the periodicity of the histone genes was influenced by the *cdc4-3* mutation. These results suggest that *TMP1*, *CDC8*, and *CDC9* may be subject to a common control that is different from that of the histone *H2A* and *H2B* genes. On the other hand, evidence presented to date suggests that the cell cycle stage-dependent timing of the expression of *HO* is very similar to that of genes like *TMP1* rather than the histone genes *H2A* and *H2B*. That is, *HO* expression is also dependent on completion of start and does not require a functional *CDC4* product. However, a consensus sequence similar to that reported to regulate the cell cycle start-dependent expression of *HO* is not found within the DNA sequences immediately preceding these other periodic genes. This suggests that at least three different processes may elicit periodic gene expression in yeast cells.

To learn more about what factors regulate the periodic expression of these genes, we have examined the expression of *TMP1* more closely. The objectives of the experiments described in this study were (i) to determine whether periodic fluctuations in *TMP1* transcript levels were regulated by transcriptional or posttranscriptional regulatory processes and (ii) to localize the *cis*-acting regulatory elements responsible for the periodic expression of *TMP1* transcript.

## MATERIALS AND METHODS

**Strains and plasmids.** *Escherichia coli* JF1754 (18) was used routinely for screening plasmid constructions and preparing plasmid DNA. *E. coli* JM101 (19) was used for preparing single-stranded M13 recombinant DNA. The *S. cerevisiae* strain used was AH22 (25). Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (3). The yeast shuttle vector YEp13 and derivative pJM135, which contains the yeast *CYC1* gene, have been described previously (5, 18). Plasmid pRS264 is a yeast shuttle vector with 2μm circle plasmid sequences, pBR322 sequences, *LEU2* sequences, and sequences for the reporter gene *lacZ*. This plasmid has been described elsewhere (R. W. Ord, Ph.D. thesis, Concordia University, Montreal, 1987). For constructions involving YEp13, pJM135, or pRS264, recombinant plasmids were selected by transformation of JF1754 to ampicillin resistance, followed by replica plating to screen for complementation of *leuB* by the yeast *LEU2* gene which is carried on these vectors.

**Plasmid constructions.** The pertinent portions of the dif-

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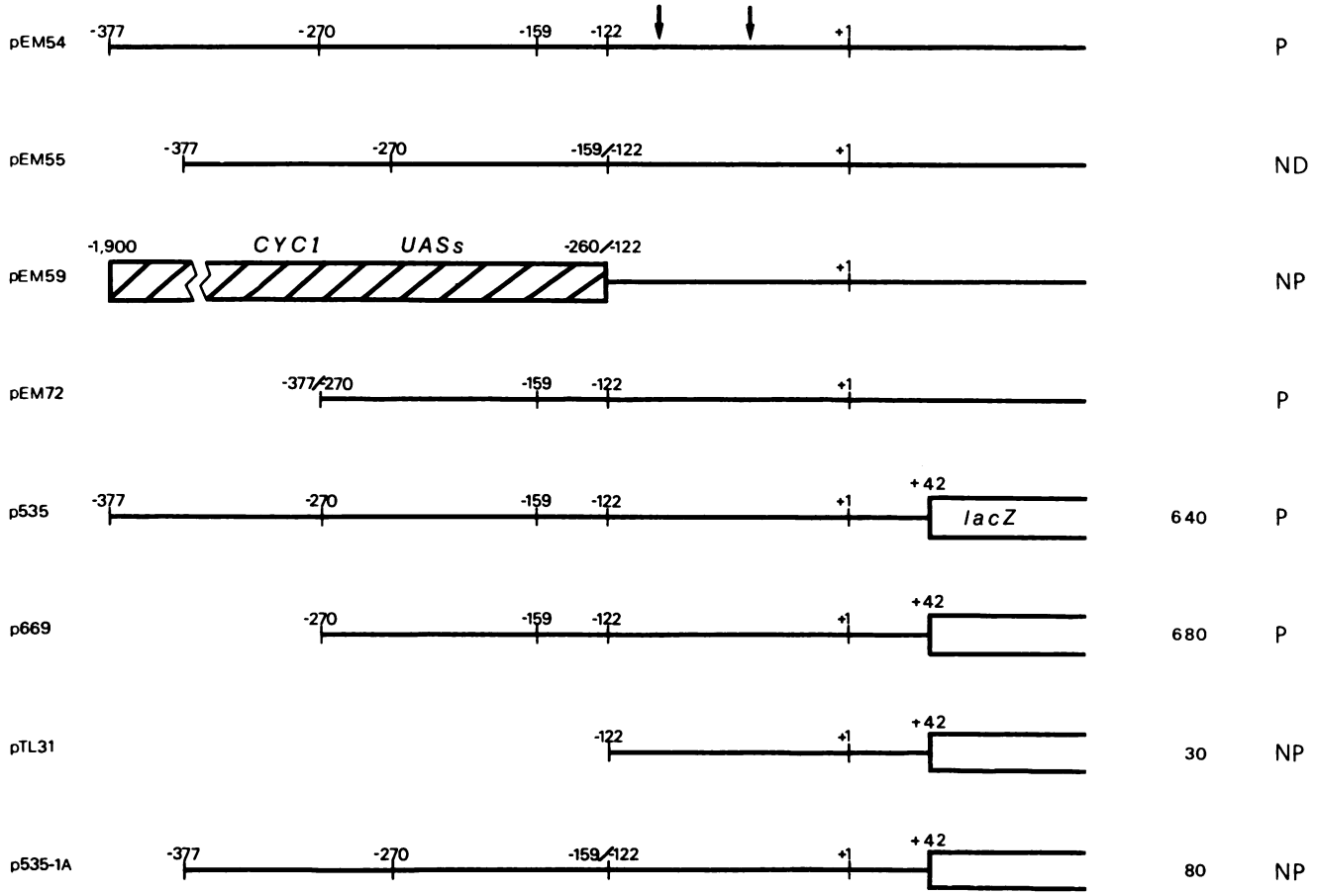


FIG. 1. Structure of the promoter regions present in the different plasmids used in this study. The constructions of the plasmids which carry these promoter regions are described in the Materials and Methods. The two vertical arrows indicate the position of the two TATA sequences within the upstream *TMP1* sequences. A single line indicates *TMP1* sequences. Regions depicted by the open box are *lacZ* sequences. Hatched segments indicate upstream sequences from the *CYC1* locus. Positions within the *TMP1* regions identified by −377, −270, −159, −122, and +42 represent the locations of the *Hin*dIII, *Pst*I, *Mlu*I, MluI, and *Sau*3A restriction endonuclease sites, respectively. Position +1 indicates the A of the first ATG of the *TMP1* ORF. Vertical lines identified by two numbers depict either the deletion of sequences (for example, in pEM55, −159/−122 indicates that sequences between −159 and −122 have been deleted) or the fusion of *TMP1* sequences with *CYC1* sequences (for example, in pEH59, −260/−122 indicates that the 3′ end of *CYC1* upstream sequences ending 260 bp upstream of the *CYC1* ATG is fused to *TMP1* sequences beginning at −122). The column to the immediate right of the promoter regions gives units (picomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per $A_{600}$ unit) of β-galactosidase activity present in log-phase cultures of yeast strain AH22 harboring each of the plasmids. The column to the far right indicates whether these promoter regions direct nonperiodic (NP) or periodic (P) gene expression. ND indicates that periodicity was not determined.

ferent promoter regions used to study *TMP1* expression are shown in Fig. 1. Plasmid pTL830 contains the entire *TMP1* coding region on a 2.7-kilobase-pair (kb) *Hin*dIII to *Bgl*II fragment (26) cloned into the *Hin*dIII and *Bam*HI sites of pUC9. From this plasmid the entire yeast insert can be easily retrieved by cleavage with *Hin*dIII and *Sma*I, the latter enzyme cutting adjacent to the *Bam*HI-*Bgl*II fusion in the pUC9 polylinker of pTL830. Plasmid pEM54 was constructed by cloning the 2.7-kb *Hin*dIII to *Sma*I fragment of pTL830, containing *TMP1*, into the large *Hin*dIII to *Pvu*II fragment of the yeast shuttle vector YEp13.

Plasmid pEM55 was constructed in two steps. First, the DNA between the two *Mlu*I sites immediately upstream of the *TMP1* coding sequence was deleted by digesting pTL830 with *Mlu*I, followed by ligation, transformation, and screening ampicillin-resistant JF1754 colonies for the appropriate construct, which was designated pEM49. Next, the *TMP1*-containing 2.7-kb *Hin*dIII to *Sma*I fragment of pEM49 was cloned into the *Hin*dIII and *Pvu*II sites of YEp13 exactly as described above.

Plasmid pEM59, which is also a YEp13 derivative but contains a *CYC1-TMP1* gene fusion, was also constructed in two steps. First, a *Hin*dIII linker was added to the *Sma*I site in pTL830. Next, the 2.3-kb *Mlu*I (site closest to the *TMP1* open reading frame [ORF]) to *Hin*dIII fragment of this plasmid was cloned into the large *Mlu*I to *Hin*dIII fragment of pJM135 (18). This construct is a YEp13 derivative which places the *TMP1* transcription initiation region immediately adjacent to the *CYC1* upstream activation sequences (UASs). However, the *CYC1* sequence on pEM59 does not include any of the *CYC1* TATA boxes (Fig. 1).

Plasmid pEM72 is identical to pEM54 except for a deletion of the DNA between the *Hin*dIII and *Pst*I sites immediately upstream of the *TMP1* ORF. It was also constructed in two steps. First, plasmid pTL830 was digested with *Hin*dIII and *Pst*I, and the ends were rendered flush with T4 DNA

polymerase (15). The resulting blunt-ended molecules were ligated in the presence of *Hin*dIII linkers to yield plasmid pEM70. Next, the 2.6-kb *Hin*dIII to *Sma*I fragment of pEM70 was cloned into the large *Hin*dIII to *Pvu*II fragment of YEp13 exactly as described above.

Plasmid pRS535 (Fig. 1) was derived from plasmid pRS264 (Ord, Ph.D. thesis) and pTL221 (25) by inserting the *Hin*dIII to *Sau*3A fragment of the *TMP1* gene (Fig. 1) into the large *Hin*dIII to *Bam*HI fragment of pRS264.

Plasmid pRS669 was constructed by removing the small *Pst*I fragment containing about 100 bp of *TMP1* upstream sequences and about 275 bp of 2μm sequences from plasmid pRS535 (Ord, thesis).

Plasmid pRS535-1A was constructed in two steps. First, the small *Mlu*I fragment of the *TMP1* gene upstream region between positions −159 and −122 was deleted from plasmid pTL221. Then the *Hin*dIII to *Sau*3A portion of this plasmid was inserted into the large *Hin*dIII to *Bam*HI fragment of pRS264.

Plasmid pTL31 was constructed in two steps. First, the *TMP1* information between the *Fnu*DII site at −122 and the *Bgl*II site at +339 was inserted into the *Bam*HI and *Hin*cII sites of puc9. The desired plasmid was screened for by using restriction endonuclease mapping and was then verified by DNA sequencing. Once constructed, the *Hin*dIII to *Sau*3A *TMP1* region of this plasmid was inserted into the large *Hin*dIII to *Bam*HI fragment of plasmid pRS264.

**Transformations.** Yeast strain AH22 was transformed by the LiCl method of Ito et al. (13). *E. coli* strains were transformed by the $CaCl_2$ procedure (15).

**Synchrony.** Synchronous cultures of AH22 transformants were prepared by the α-factor release method (21) with the following modifications. Cultures were grown overnight in 200 ml of leucine omission medium to an $A_{600}$ of 0.2. The medium was then adjusted to 10 mM citrate (pH 4) and 200 μg of bovine serum albumin per ml. One milligram of synthetic α-factor was dissolved in 0.5 ml of methanol and added to the culture (volume to flask ratio of 1 to 5). After 2.5 h of incubation, most of the cells were arrested in G1, as indicated by the lack of budded cells. The arrested cells were harvested by centrifugation, washed three times with 5 ml of water, and then resuspended in 200 ml of fresh leucine omission medium. Samples of the culture (15 ml) were harvested at 10-min intervals by rapid filtration as described previously (17). The quality of the synchrony and progression through the cell cycle were monitored by determining the proportion of unbudded cells present at different times following release from α-factor-induced arrest.

**RNA isolation.** Total cellular RNA was isolated as described previously (17) with the following modifications. RNA extractions were performed with a phenol-chloroform-isoamyl alcohol mixture (25:24:1) containing 1% sodium dodecyl sulfate (SDS) and 0.3 M sodium acetate (pH 5.2) to exclude DNA. RNA was ethanol precipitated with ammonium acetate (2.5 M final) instead of sodium acetate. Polyadenylated [poly(A)$^+$] RNA was isolated by a single passage of total cellular RNA over an oligo(dT)-cellulose column as described previously (17).

**Primer extension analysis.** RNA analysis by primer extension was performed as described previously (18) with the following modifications. One picomole of primer was end labeled with $^{32}P$ (10 μCi) in a total volume of 20 μl (15). After the kinase was inactivated (5 min at 65°C), 1 μl of labeled primer was mixed with 5 to 10 μg of RNA in 10 μl of reverse transcriptase buffer (100 mM Tris hydrochloride [pH 8.5], 100 mM MgCl, 100 mM KCl, 1 mM each dATP, dGTP, dCTP, and dTTP, 0.2 mM dithiothreitol) and allowed to anneal first by heating (5 min at 85°C) followed by cooling on ice (10 min); then 0.5 U of avian myeoblastosis virus reverse transcriptase was added to each tube, and the reaction mixes were incubated at 42°C for 1 h. Each reaction was terminated by adding 11 μl of alkaline stop buffer (18), and RNA-DNA hybrids were denatured (2 min at 100°C, followed by 10 min on ice). A 3-μl amount of each reaction mixture was then electrophoresed on a short (16 cm) 8% polyacrylamide–7 M urea sequencing gel run at 500 V for 2 h.

**Northern hybridizations.** RNA was denatured by heat treatment (2 min at 85°C) in the presence of loading buffer (0.05% bromphenol blue, 0.05% xylene cyanol, 6% glycerol, final concentration), loaded immediately onto an 0.8% (wt/vol) agarose gel, and electrophoresed in TEB buffer (15) at 100 V for approximately 2 h. Following electrophoresis, the gel was dried and prepared for hybridization as described by Tsao et al. (27). Dried gels were prehybridized in 10 ml of hybridization buffer (6× SSC [15], 0.1% sodium pyrophosphate, 0.5% autoclaved SDS, 100 μg of tRNA per ml, 0.5 mg of heparin) for 2 to 4 h at 50°C. Hybridization was performed by adding 1 pmol of $^{32}P$-labeled oligonucleotide directly to the prehybridization solution and incubating overnight at 50°C. Following hybridization, the gel was washed in 250 ml of 2× SSC–0.5% sodium pyrophosphate four times at room temperature for 5 min each, once at 37°C for 1 h, and once at 50°C for 10 min.

**β-Galactosidase assays.** β-Galactosidase assays of yeast transformants were performed with Brij-permeabilized cells (25).

**Oligonucleotides, enzymes, and other reagents.** Synthetic oligonucleotide P20 (5′-GCATCGATAGCAGCACCA-3′), which hybridizes to *LEU2* mRNA, was kindly provided by Bryan McNeil (University of Toronto). Oligonucleotides P11 (5′-GTGCCTGTTCTATCTGGCCT-3′) and P12 (5′-AGA CTCAACGTACCAAGTGCC-3′), which hybridize to *TMP1* mRNA, were obtained through David Thomas (NRC Biotechnology Research Institute, Montreal). All DNA restriction and modification enzymes were purchased from Pharmacia, Uppsala, Sweden. Agarose was from International Biotechnologies, Inc., New Haven, Conn., and heparin (grade I) and synthetic α-factor were purchased from Sigma Chemical Co., St. Louis, Mo.

**Nomenclature.** Nucleotides upstream of the first *TMP1* translation start codon are numbered decreasingly with negative integers. The A of the start codon is +1, and all downstream points are numbered increasingly with positive integers.

## RESULTS

**Mapping the *TMP1* mRNA 5′ ends.** The 5′ ends of the *TMP1* transcripts were mapped by the primer extension method with synthetic oligonucleotides complementary to the sense strand of *TMP1* between +78 and +98 (primer P11) and between +94 and +113 (primer P12). Since *TMP1* is normally expressed at very low levels (8, 25), we performed the analysis with total cellular RNA isolated from strain AH22 transformed with a high-copy-number plasmid carrying the *TMP1* gene (plasmid pEM54) and also with poly(A)$^+$-enriched AH22 RNA. The results of the extension analysis are shown in Fig. 2. Lanes 1, 3, and 5 show the extension products with poly(A)$^+$ RNA, while lanes 2, 4, and 6 represent total RNA isolated from the transformant. Two different primers were used for cDNA synthesis (P12 in lanes 1 to 4, P11 in lanes 5 and 6) to facilitate the mapping of

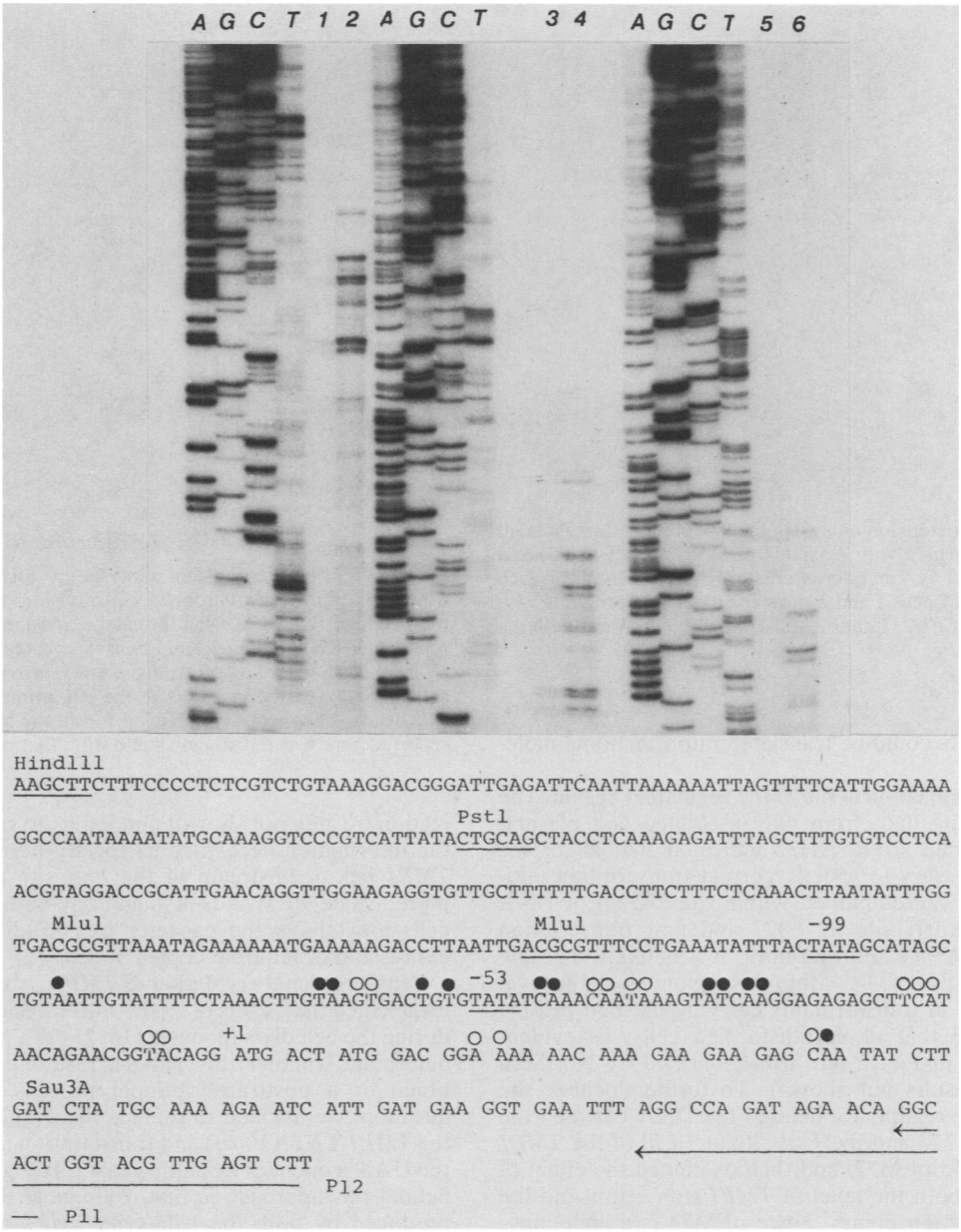


FIG. 2. Mapping the 5′ ends of *TMP1* mRNA by primer extension. Oligonucleotides P11 and P12 were end labeled with $^{32}P$, hybridized to poly(A)$^+$ or total cellular mRNA, and extended with reverse transcriptase. The extension products were then resolved on the sequencing gel prior to autoradiography. Lanes 1 through 4 represent oligonucleotide P12 extension products with either poly(A)$^+$ (lanes 1 and 3) or total cellular RNA from an AH22(pEM54) transformant (lanes 2 and 4). The samples in lanes 3 and 4 were electrophoresed for a longer period (the same time as samples in lanes 5 and 6) in order to achieve better resolution of the most-upstream ends. The extension products were run alongside a sequencing ladder of the *TMP1* sense strand, which was primed with P12. The poor quality of the bands in the T lane is an artifact of the P12 primer. Lanes 5 and 6 represent extension products of the P11 primer with poly(A)$^+$ and total cellular RNA from an AH22(pEM54) transformant, respectively. The sequencing ladder adjacent to lanes 5 and 6 is the *TMP1* sense strand primed with P11. The estimated location of the major (●) and minor (○) transcription initiation points are indicated above the *TMP1* DNA sequence. The positions of relevant restriction sites, the two potential TATA boxes, and the P11 and P12 hybridization sites are also indicated.

transcript ends alongside sequencing ladders of *TMP1* and to ensure that the results were consistent for different primers. From the results shown, it is evident that *TMP1* exhibited multiple transcription initiation points, some of which lay within the coding region of the gene (Fig. 2). The pattern of 5′ ends observed in the experiment was generally consistent for the two primers and also for poly(A)$^+$ versus plasmid-amplified RNA. Heterogeneity in transcript 5′ ends is common for yeast mRNAs (17, 18), and so our findings for *TMP1* are not unusual. Notably, each of the *TMP1* transcript ends lay downstream of a TATA sequence at −99 (Fig. 2). From other results described below, it seems likely that this TATA is involved in selecting most of the transcription initiation points. A second TATA sequence which is further downstream at −53 may be fortuitous and responsible for the initiation of transcripts within the coding region. On the basis of amino acid sequence similarity between various thymidylate synthases (26), it is highly unlikely that these

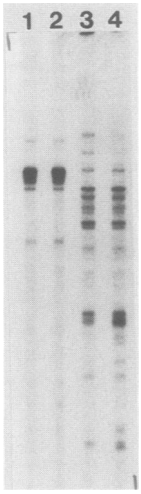


FIG. 3. Primer extension analysis of total cellular RNA isolated from AH22 transformed with plasmids pEM54 (lanes 1 and 3) and pEM72 (lanes 2 and 4). Ten micrograms of RNA was used for each extension reaction. Lanes 1 and 2 show the extension products with the P20 primer for *LEU2*. Lanes 3 and 4 show the products with the P12 primer for *TMP1*.

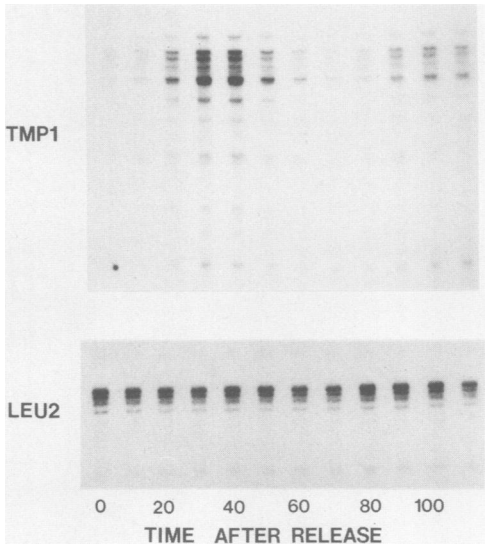


FIG. 4. Primer extension analysis of pEM72-directed *TMP1* transcripts during synchronous cell division. Five micrograms of total cellular RNA was used for each extension reaction. Each lane represents RNA isolated from cells harvested at 10-min intervals after release from α-factor-induced G1 arrest. The lower panel shows the extension products of the P20 primer for *LEU2* mRNA. The degree of synchrony achieved for all the synchronous cultures reported here was indistinguishable from that represented in Fig. 8.

smaller transcripts could be translated into functional molecules.

**Preliminary localization of the *TMP1* regulatory region.** The transcript mapping data from untransformed and plasmid pEM54-transformed strain AH22 indicated that all of the information necessary to specify correct transcription initiation for *TMP1* was contained within the 290 bp of DNA between the *Hin*dIII site at −377 and first transcription initiation point at −86. Furthermore, it was likely that any UAS activity would also lie within this region, since the level of *TMP1* mRNA in transformants carrying plasmid pEM54 greatly exceeded that of untransformed cells, as evident from the results in Fig. 2 but also confirmed by Northern hybridization (results not shown). To further localize the *TMP1* regulatory region, we deleted the DNA between the *Hin*dIII site at −377 and the *Pst*I site at −270 of the *TMP1* sequence (plasmid pEM72) and then examined the effect of this deletion on both the level of *TMP1* transcripts and the selection of initiation points. Since pEM72 is a high-copy-number vector and identical to plasmid pEM54 except for the deletion, we assayed total cellular RNA isolated from an AH22(pEM72) transformant. The results of this experiment revealed that, relative to the pEM54 control, removal of the *Hin*dIII to *Pst*I region of *TMP1* did not affect either the level of *TMP1* mRNA or the selection of transcription initiation points (Fig. 3).

To ensure that all of the information necessary to specify periodic expression was contained on plasmid pEM72, we followed the relative levels of *TMP1* mRNA during synchronous cell division of AH22 transformed with this plasmid. The contribution of the chromosomal *TMP1* gene to the level of transcripts seen for this experiment was negligible due to the high copy number of pEM72. This was also evident from the results presented below, when *TMP1* was placed under control of the *CYC1* UASs. The results of primer extension analysis demonstrated that levels of all pEM72-directed *TMP1* transcripts fluctuated periodically relative to the mRNA levels expressed from the nonperiodic *LEU2* gene (Fig. 4). This result was also confirmed by Northern hybridization (results not shown) and led us to conclude that all of the information necessary to specify periodic regulation of *TMP1* lies downstream of the *Pst*I site at −270. Furthermore, since pEM72 is a high-copy-number vector, yeast cells must have the capacity to periodically regulate the expression of multiple copies of *TMP1*.

**Transcriptional regulation of *TMP1*.** Although it has been established that levels of *TMP1* mRNA fluctuate periodically during the cell division cycle (16, 25, 28), it was important to determine whether this pattern resulted from a transcriptional or a posttranscriptional process. To address this question, we decided to replace the sequences upstream of the *TMP1* TATA boxes and transcription initiation sites with the UAS sequences from the yeast *CYC1* gene. The rationale behind this approach is that if identical RNA species were produced by both the wild-type *TMP1* gene and a *TMP1* gene under the control of the *CYC1* UASs, then the RNA species encoded by both genes should be subject to the same posttranscriptional processes. Furthermore, any differences in expression between these genes would be due to their different regulatory regions. We chose the UAS regions of the yeast *CYC1* gene for three reasons. First, both the DNA sequences which constitute the *CYC1* UASs and the factors which interact with them have been well defined (18, 24). Second, since *CYC1* is involved in the oxidative phosphorylation process, it was unlikely to be subject to cell cycle-dependent regulation. Third, the level of expression dictated by *CYC1* can be easily manipulated by simple changes of the carbon source used to support cell growth (10, 11). This latter point was important because we wanted to ensure that the level of *TMP1* expression from the plasmid-borne gene with the hybrid promoter did not exceed that of the plasmid-borne wild-type *TMP1* gene. This would eliminate the possibility that lack of cell cycle regulation under this circumstance might simply represent saturation of an mRNA destabilization system.

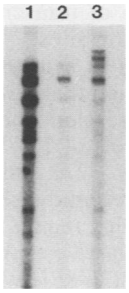


FIG. 5. Primer extension analysis of the *CYC1-TMP1* gene fusion. $^{32}P$ end-labeled P12 primer was hybridized with 10 μg of total cellular RNA isolated from an AH22(pEM59) transformant grown on either 2% glycerol plus 2% ethanol (lane 1) or 2% glucose (lane 2) and extended with reverse transcriptase. Lane 3 shows the extension products with 10 μg of total cellular RNA isolated from an AH22(pEM54) transformant and represents the native *TMP1* gene.

*TMP1* transcription was placed under control of the *CYC1* UAS region by constructing plasmid pEM59 as described in the Materials and Methods section. In plasmid pEM59, the *CYC1* UAS region, excluding the *CYC1* TATA boxes (18), is placed adjacent to and within approximately 50 bp of the most upstream *TMP1* transcription initiation point and the potential TATA box at position −99 (Fig. 1 and 2). To demonstrate that *TMP1* expression was governed by the *CYC1* UAS region on pEM59, we examined the induction of *TMP1* transcripts under inducing (growth on glycerol plus ethanol) and repressing (growth on glucose) conditions for *CYC1* gene expression. The results of the primer extension analysis are shown in Fig. 5. Lane 1 represents *TMP1* mRNA levels in AH22 transformed with pEM59 when *CYC1* expression was induced by growth on glycerol and ethanol. Lane 2 shows that the production of *TMP1* mRNA in the transformant was severely reduced by growth on glucose relative to that of the induced cells and the native *TMP1* gene on pEM54 (lane 3). By comparison of lanes 1 and 2, it is evident that the *TMP1* gene was controlled by the *CYC1* UAS region on plasmid pEM59, since *TMP1* transcripts were both induced and repressed by carbon sources which similarly affected *CYC1* expression. It is also evident from these data that, although the *CYC1* UAS region influenced the relative frequency at which the various transcription initiation points were used, it did not greatly alter the choice of initiation sites. In Fig. 5, it appears that the most 5′ *TMP1* transcription initiation points were eliminated by the presence of the *CYC1* UAS region; however, in other primer extension analyses (for example, see Fig. 6), it is evident that these sites were used but at a much reduced frequency.

To address the question of transcriptional versus posttranscriptional control, we determined the effect of the *CYC1* UAS region on the production of *TMP1* mRNA during synchronous cell division of a pEM59 transformant grown on glucose. The degree of synchrony exhibited by the culture for this experiment was virtually identical to that of the pEM72 synchrony (Fig. 4). The results of the primer extension analysis are shown in Fig. 6. The levels of pEM59-directed transcripts remained relatively constant throughout synchronous cell division. By comparison with the *LEU2* control, it is evident that the reduced levels of *TMP1* mRNA seen at the 0 and 10-min time points were not the result of a simple loading error. This does not represent true periodic behavior, however, since a similar pattern was not seen in the second synchronous G1-S phase interface between 80 and 100 min after release from α-factor arrest. By analysis of

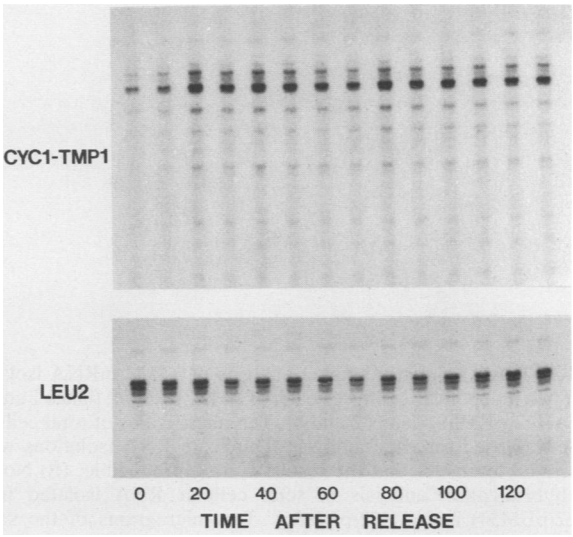


FIG. 6. Primer extension analysis of *TMP1* mRNA during synchronous cell division of an AH22(pEM59) transformant. The analysis was performed for *TMP1* and *LEU2* mRNA as described in the text, with 5 μg of total cellular RNA from each time point.

β-galactosidase activity in AH22 transformed with a *CYC1-lacZ* gene fusion, we found that α-factor-induced G1 arrest had a negative effect on *CYC1* gene expression (data not shown). Therefore, the reduced levels of *TMP1* mRNA seen at these early time points are probably due to the fortuitous effect of α-factor treatment on the *CYC1* region used in this study. We also found that *TMP1* mRNA levels expressed by the pEM59 transformant grown with raffinose as an inducing carbon source were not periodic, although they were dramatically elevated relative to levels in the glucose-grown cells. These results also confirm that the contribution of the chromosomal *TMP1* gene to the periodic pattern of *TMP1* mRNA accumulation observed for pEM72 (Fig. 4) is negligible since (i) when grown on glucose, the *TMP1* mRNA levels in pEM72 transformants exceeded that of pEM59 transformants (compare Fig. 3 and 5) and (ii) the expression of the chromosomal *TMP1* gene was clearly insufficient to produce a periodic pattern of *TMP1* mRNA accumulation in a synchronized pEM59 transformant (Fig. 6). The observation that full-length *TMP1* transcripts were produced at relatively constant levels throughout the cell cycle of a pEM59 transformant is strong evidence that transient changes in mRNA stability do not account for periodic expression of the *TMP1* gene. Therefore, the periodic expression of *TMP1* transcript must be primarily due to periodic changes in transcription.

**Expression of *TMP1′-′lacZ* gene fusions in asynchronous cultures.** The results described above, which showed that periodic expression resulted from periodic changes in transcription and suggested that the regulatory region responsible for periodic expression was upstream, were confirmed and extended by analyzing a series of *TMP1′-′lacZ* gene fusions. This approach illustrated that the regulatory region of *TMP1* could also confer cell cycle-dependent gene expression on a foreign gene, the *E. coli lacZ* gene. The series of gene fusions used are illustrated in Fig. 1, and their construction is described in the Materials and Methods section. Initially, we simply examined the levels of β-galactosidase activity corresponding to each derivative in exponentially growing cells (Fig. 1). Removal of the *Hin*dIII to *Pst*I region

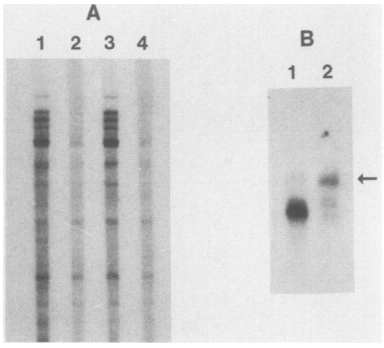


FIG. 7. (A) Primer extension analysis of *TMP1* mRNA isolated from two independent cultures each of AH22(pEM54) (lanes 1 and 3) and AH22(pEM55) (lanes 2 and 4). Ten micrograms of total cellular RNA was used for each analysis. Duplicate RNA isolations were performed to ensure that the results were reproducible. (B) Northern hybridization analysis of total cellular RNA isolated from AH22(pEM54) and AH22(pEM55). Ten micrograms of the same RNA used for the primer extension analysis shown in panel A was also used for the hybridization. The arrow indicates the position of the 1.7-kb RNA species.

to generate pRS669 did not produce any significant effect on *lacZ* expression relative to the complete upstream region on plasmid pRS535 (Fig. 1). Removal of information between −377 and −122, however, resulted in a severe drop (approximately 20-fold) in *TMP1'-'lacZ* expression. Similarly, deletion of the 37-bp region between positions −159 and −122 resulted in about an eightfold reduction in β-galactosidase expression. These results showed that the −270 to −122 and to a lesser extent the smaller −159 to −122 region played a positive role critical for normal levels of *TMP1* expression. Furthermore, since this region was also upstream of the transcription start sites, it appears to contain sequences necessary for UAS(s) activity (9).

To learn more about the possible role of this putative UAS region, we examined the effect of the *Mlu*I deletion on the level of transcription of the native *TMP1* gene. Plasmid pEM55 was constructed for this purpose and is identical to plasmid pEM54 except for the deletion of the information between the two *Mlu*I sites. Total RNA isolated from two independent AH22(pEM55) transformants was analyzed by primer extension. The results of this experiment are shown in Fig. 7A (lanes 2 and 4) alongside extension products with RNA from an AH22(pEM54) transformant (lanes 1 and 3) and confirmed that removal of this region caused a reduction in the level of *TMP1* transcripts which exhibited normal 5′ ends. Longer extension products were seen for the pEM55 transformants, as indicated by background smear corresponding to higher-molecular-weight species (Fig. 7, lanes 2 and 4). This suggested that removal of the *Mlu*I region resulted in larger transcripts reading through the normal region of *TMP1* transcription initiation. These results were confirmed by northern analysis of total cellular RNA from pEM54 and pEM55 transformants (Fig. 7B). This analysis showed that levels of normal *TMP1* transcripts were reduced; however, there was an increase in the amount of a larger transcript for the pEM55 transformant. The size of this transcript (approx. 1.7 kb), which was also seen to a lesser extent in the pEM54 transformant, indicates that it initiated within 2μm DNA sequences which were immediately upstream of the *Hin*dIII site in both of these plasmids. Thus, it appears that the presence of the *Mlu*I to *Mlu*I region not only influence the level of *TMP1* mRNA but may also act to inhibit the production of transcripts which may initiate fortuitously at far upstream sites.

***TMP1'-'lacZ* expression in synchronous cultures.** The results described above found that *cis*-acting information necessary for periodic expression and a positive element(s) necessary for normal levels of *TMP1* expression were contained between positions −270 and −122. Furthermore, a positive element must overlap the region between −159 and −122, since deletion of this region reduced expression. To determine whether this positive element was responsible for the periodic expression of *TMP1*, we followed *lacZ* expression in AH22 transformants harboring pRS535, pRS669, and pRS535-1A. The results obtained demonstrate that both pRS535 (data not shown) and pRS669 expressed *lacZ* periodically, while pRS535-1A directed the nonperiodic expression of *lacZ* (Fig. 8). That is, the steplike pattern of β-galactosidase activity observed for the pRS669 transformant was typical of the pattern expected for a periodically synthesized stable protein, while in the pRS535-1A transformant β-galactosidase levels were severely reduced and increased in a linear fashion typical of nonperiodic synthesis. These results are therefore consistent with the previous results showing that deletion of this region (−159 to −122) severely reduces transcription of *TMP1*. Furthermore, these results indicate that this region is critical for the periodic transcription of *TMP1*. Therefore, the *cis*-acting positive element which overlaps positions −159 and −122 is also necessary for the periodic expression of *TMP1*.

## DISCUSSION

The results presented in this study demonstrate that the periodic nature of *TMP1* mRNA levels during the *S. cerevisiae* cell cycle is governed primarily at the level of transcription and does not involve, to any great extent, transient changes in the stability of *TMP1* mRNA. In this respect the regulation of *TMP1* in yeast cells differs markedly from that of the human thymidylate synthase gene. That is, although levels of the human thymidylate synthase transcript also fluctuate in a cell cycle stage-dependent fashion, it is transient changes in transcript stability, not transcriptional regulation, which govern its periodic expression.

Although transcript stability apparently does not play a role in regulating thymidylate synthase expression in yeast, it has been found that in yeast cells, as in human cells, a posttranscriptional process plays a significant role in thymidylate synthase regulation. However, in yeast cells this additional level of regulation is posttranslational and affects the stability of thymidylate synthase activity (8). This instability results in activity being lost when cultures enter the stationary phase, when cultures are arrested in G1 phase of the cell cycle, and following S phase of the cell cycle in synchronous cultures.

Expression analysis of the *TMP1* gene and several derivatives with altered 5′ sequences enabled us to localize the sequences controlling periodic expression to a 150-bp region immediately upstream of the transcription initiation sites. These results were verified by demonstrating that the periodic nature of *TMP1'-'lacZ* fusion gene expression was also dependent on this 150-bp sequence. Furthermore, based on the following arguments, we believe that the upstream sequences required for periodic expression encode an element having the properties of a periodic UAS (9) and that sequences critical for this UAS activity are found between positions −122 and −159. (i) Deletion of this 37-bp region resulted in both a severe reduction in expression and the loss

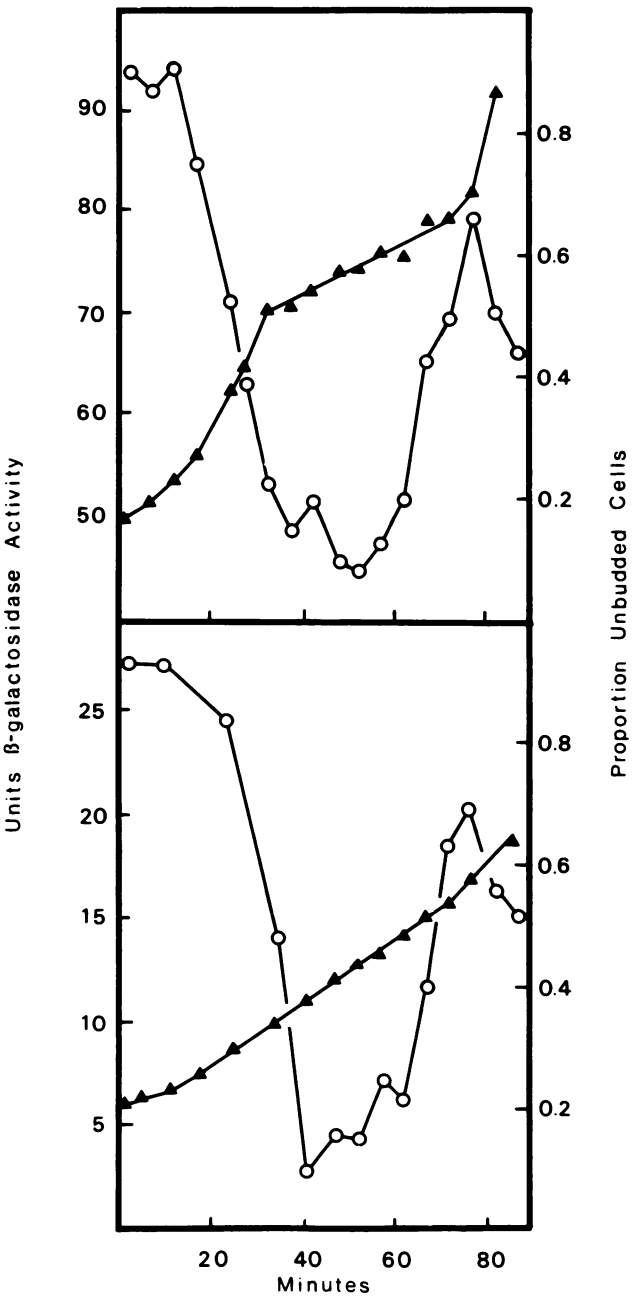


FIG. 8. Expression of β-galactosidase activity (▲) during synchronous growth of AH22 transformants harboring pRS669 (upper panel) and pRS535-1A (lower panel). Synchronous cultures were generated by the α-factor arrest release method as described in the Materials and Methods section. Units of β-galactosidase are expressed as picomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milliliter of culture; the values plotted are the means of duplicate samples. These duplicate samples did not vary from their means by more than 3%. Also included to indicate the quality of the synchrony and the cell cycle stage are the proportion of unbudded cells (○). The abscissa indicates minutes following release from α-factor-induced G1 arrest. This experiment was also performed with an AH22 derivative harboring this fusion gene integrated at the *leu2* locus, and although levels of expression were reduced, a steplike profile indistinguishable from the p669 profile was obtained (data not shown). In the experiment with the integrated fusion gene, samples were collected up to 100 min, and activity levels continued to increase in a linear fashion, as defined by five data points for 20 min following the inflection point at 80 min.

of periodic expression. (ii) This region is located upstream of both the transcription start sites and potential TATA sequences. (iii) Replacement of this region with the UASs from the yeast *CYC1* gene resulted in a nonperiodically transcribed *TMP1* gene which was regulated like the *CYC1* gene. (iv) The periodic expression of a foreign gene (*lacZ*), which was placed under control of *TMP1* regulatory sequences, became dramatically reduced and nonperiodic when this region was altered by deletion.

The presence of a positive element important for periodic expression is similar to the promoter elements defined for the *S. cerevisiae H2A-H2B* gene pair and the *HO* gene (4, 22). However, the regulation of *TMP1* transcription is clearly distinct from that of the histones both in the timing of induction after release from G1 arrest and in the sense that it is independent of the *CDC4* gene product (28).

Computer-assisted analysis of the DNA sequences immediately preceding the *TMP1*, *CDC8*, and *CDC9* genes, all of which exhibit a similar pattern of periodic expression during the cell cycle (28), found the occurrence of at least two 5′-TPuACGCGTN(T/A)-3′ consensus elements, where Pu is a purine and N is any nucleotide, within 200 bp of the translation start codon of all these genes. For the *TMP1* gene, the center of this element is the recognition site for restriction endonuclease *Mlu*I; therefore, the construction of p535-1A generated a promoter with the 37 bp between the two deleted *Mlu*I sites but possessing one hybrid version of this element. Since p535-1A did not direct periodic expression, a single hybrid copy of this element is apparently not sufficient for periodic expression. It is of interest, however, that for both *TMP1* and *CDC9*, this sequence is contained within a dyad symmetry (2, 26). The similar positioning of 5′-TPuACGCGTN(T/A)-3′ element has been found for the *S. cerevisiae* DNA topoisomerase II gene (7). This suggests that a DNA sequence possessing dyad symmetry and containing this element may be a feature shared by some cell cycle-regulated genes in *S. cerevisiae*. These similarities and our results showing that *TMP1* is regulated at the level of its transcription suggest that the periodic expression of other genes involved in DNA metabolism, such as those encoding the subunits of ribonucleotide reductase (6), may also be controlled at the level of their transcription.

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