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The Cell Cycle Stage and Batch Culture Stage Dependent Expression of the Thymidylate Synthase Gene (TMP1) in *Saccharomyces cerevisiae*

Evelyne M. Calmels

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ABSTRACT

The Cell Cycle Stage and Batch Culture Stage Dependent Expression of the Thymidylate Synthase Gene (TMP1) in *Saccharomyces cerevisiae*

Evelyne M. Calmels

I investigated the cell cycle stage and batch culture stage dependent expression of the *TMP1* gene. The levels of *TMP1* mRNA were monitored at different stages of the cell cycle and at the different stages of batch culture growth. The major findings were:

1) The levels of *TMP1* mRNA were low in cells subjected to nutrient limitation (a stationary phase condition) or exposed to the pheromone alpha-factor (G1 arrest).

2) The periodic increase of *TMP1* mRNA levels occured early after the execution of "START" prior to the *CDC4* mediated event of the cell cycle.

3) The induction of *TMP1* transcription in cultures released from stationary phase did not require *de novo* protein synthesis.

4) An increase in the *TMP1* gene dosage resulted in a proportional increase in *TMP1* mRNA levels; therefore yeast did not compensate for extra copies of the *TMP1* gene at the transcriptional level.

A mon Grand-Père

Louis CALMELS

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INTRODUCTION

In Saccharomyces cerevisiae the TMP1 gene (Taylor et al.,1982) encodes the enzyme thymidylate synthase (T.S.) (EC 2.1.1.45). T.S. catalyzes the reductive methylation of depxyuridylic acid to form thymidylic acid. The TMP1 gene product catalyzes the only de novo pathway of dTMP biosynthesis in yeast. Perturbations of yeast intracellular pools of dTMP lead to either thymidineless death (Toper et al.,1981), or thymidylate excess death (Barclay et al.,1978). These observations suggest that the expression of the TMP1 gene must be precisely regulated in order to maintain non lethal intracellular levels of dTMP. Previous studies have demonstrated that the levels of TMP1 mRNA fluctuate in a cell cycle-dependent fashion (Storms et al.,1984). TMP1 mRNA levels are maximal in late G1 near the beginning of DNA synthesis (S phase).

Regulatory mechanism(s) which control the periodic expression of eukaryotic genes encoding S phase specific proteins.

The cell cycle stage dependent expression of genes encoding proteins required for chromatin replication has been observed in a variety of eukaryotic cells. These periodically expressed genes include: the mouse cell line

3T6 dihydrofolate reductase (DHFR) (Farnham et al.,1985), the human, mouse, and yeast histones (reviewed by Schumperli 1986), and the mouse cell line 3T6 thymidylate synthase and thymidine kinase (Navalgund et al.,1980). The mRNA levels encoded by all these genes periodically increase near the onset of S phase.

The following examples illustrate some of the regulatory mechanisms postulated to control the periodic expression of eukaryotic genes whose products are required for chromosome replication. Virtually all these studies suggest that the periodic induction of these genes is initially controlled by a mechanism which modulates the rate of RNA transcription in a cell-cycle stage dependent manner.

Jin-Shyun et al.,(1982) showed that the expression of the DHFR gene in the mouse cell line 3T6 is primarily regulated at the level of transcription. The increase in the levels of DHFR mRNA at the onset of S phase, is due to a proportional increase in the rate of transcription.

Several other studies have shown that the mechanism(s) which regulate(s) the periodic expression of proteins involved in chromatin replication include both transcriptional and posttranscriptional events. Often, posttranscriptional events play important roles in regulating periodic expression. The following findings will illustrate regulatory mechanisms which operate mainly

at the posttranscriptional level.

Lucher et al.(1985) showed that posttranscriptional control plays an important role in the regulation of histone 4 (H4) in mouse cells. They found that mouse histone H4 mRNA levels in exponentially growing cells are 120 times higher than the levels found in G1 arrested mouse cells. Furthermore, they demonstrated that the rate of H4 RNA synthesis only increases by a factor of 3 in exponentially growing cells as compared to quiescent cells. This suggests that H4 mRNA levels are primarily regulated by a posttranscriptional mechanism. To show that a posttranscriptional mechanism played a major role in the regulation of the mouse H4 gene, they studied the expression of a hybrid gene containing the early promoter of SV40 (a promoter constitutively expressed throughout the cell cycle) fused to the 3' terminal half of the mouse H4 gene. Their results revealed that the hybrid SV40-H4 gene is cell cycle regulated in mouse cells.

In addition, they demonstrated that the 3' end of the H4 message is the target for the posttranscriptional regulatory mechanism. Originally, the SV40 promoter was fused to the structural portion of the E.coli galK gene. The 3' terminal half of the H4 gene was inserted between the SV40 promoter and the galK gene. Any transcript escaping the histone specific termination site will give rise to a SV40- H4 initiated galK mRNA. A

small proportion of the SV40-H4 initiated transcripts terminated at the galK termination site. The levels of these messages, in contrast to those which terminate at the H4 termination site, are constant throughout the cell cycle. These results suggest i) that the posttrancriptional mechanism which regulates H4 gene expression is sufficient to confer periodicity to the SV40-H4 hybrid gene, ii) that the integrity of the H4 mRNA 3' end is required for posttranscriptional regulation.

Similar studies (Ross et al.,1986) on the regulation of the human histone H4 demonstrated that the 3' end of the H4 mRNA is the target for a posttranscriptional regulatory mechanism. They showed that the rapid decay of H4 mRNA, observed when DNA synthesis is blocked, is associated with an increased rate of degradation of the message. This degradation proceeds from the 3' towards the 5' end. Together, these results suggest that the periodic increase of both mouse and human H4 mRNA is predominently due to posttranscriptional regulation which involves the 3' terminus of the mRNA.

Jenh et al. (1985) have also shown that the S phase specific increase of thymidylate synthase (T.S.) mRNA, in the mouse cell line 3T6, could not be explained solely by an increase in the rate of transcription of the T.S. gene at the onset of S phase. Their findings revealed that the

total T.S. mRNA population is composed of a stable polyA$^+$ species and a less stable polyA$^-$ species. They showed that 70% of the T.S. message is polyadenylated in S phase cells. In contrast, in quiescent cells, which have low levels of T.S. message, only 30% of the T.S. message is polyadenylated. They postulate that differential polyadenylation of T.S. mRNA during the cell-cycle could be the major mechanism regulating the periodic levels of T.S. mRNA in the mouse cell line 3T6. This mechanism would increase the T.S. mRNA levels at the onset of S phase by increasing the proportion of the stable polyA$^+$ species in the population.

The molecular mechanisms regulating periodic expression, have been investigated in many studies. Some of these studies present evidence supporting the periodic synthesis or activation of a "factor" which induces RNA transcription. Heintz et al.(1984) showed that S phase nuclear extracts from human cell lines contain a trans-acting "factor" that activates the transcription of the human H4 gene (using an *in vitro* system). Furthermore, recent studies (Capasso et al.,1985) have revealed that the trans-acting S phase "factor" is specific *in vivo*. They found that the presence of many copies of the H4 gene apparently sequesters a limiting "factor" which is required for the induction of H4 transcription. This "factor" is specific for H4

induction, since the periodic transcription of other human histone genes is not affected by the excess copies of the H4 gene.

Similarly, investigations of T.S. and thymidine kinase (T.K.) gene expression in mouse 3T3 cells, indicate that the induction of these 2 genes is dependent on the accumulation of a "factor" synthesized during the late G1 interval (Coppock et al.,1985). The induction of the T.S. and T.K. genes is prevented when quiescent 3T3 cells are stimulated to grow in the presence of cycloheximide. Pulse studies, with cycloheximide, suggest that the accumulation of a labile protein in the late G1 interval is required to induce the transcription of these 2 genes. They suggested that a common regulatory mechanism might be responsible for the periodic increase in the transcriptional rates of both the T.K. and T.S. genes.

Stimac et al.(1984) have showed that the cell cycle dependent increase of the human histone, H3 , mRNA levels is controlled by a labile protein whose synthesis is required during the non S phase intervals to maintain low levels of H3 mRNA. The addition of cycloheximide to a non S phase synchronized population (these cells have low levels of H3 mRNA), results in a 22 fold increase in the mRNA levels. Cycloheximide added to S phase cells results only in a 4 fold increase in mRNA levels relative to levels found in untreated S phase cells. These results

suggest that the synthesis of a labile protein is required to maintain low levels of mRNA during the non S phase intervals in human cell.

These examples of periodic gene regulation are not consistent with a universal regulatory mechanism controlling the periodic expression of these genes. However, the diverse mechanisms regulating periodic expression do not preclude the possibility that some of these genes might share common transcriptional and/or posttranscriptional regulatory mechanisms.

Experimental design

The investigations presented in this study have attempted to answer the following questions;

i) Was TMP1 encoded mRNA polyadenylated? To answer this question I compared the levels of TMP1 transcript in total RNA and in polyA$^+$ RNA.

ii) Where did the 5' end of the TMP1 transcript begin? The primer extension method was used to determine the initiation sites of TMP1 transcription.

iii) Were the levels of TMP1 mRNA growth rate dependent? To show whether the levels of TMP1 mRNA were growth rate dependent I quantitated the levels of TMP1 mRNA in total RNA isolated from yeast cultures grown in super-rich and minimal media.

iv) What happens to the TMP1 mRNA levels when yeast

cells are arrested in the G1 phase of the cell cycle? Two methods were used to obtain G1 cells. One method arrested cells in stationary phase. The other method arrested cells with the pheromone alpha-factor. In yeast nutrient limitation or exposure to the pheromone alpha-factor causes the cells to arrest at or near a hypothetical "START" event (Bucking-Throm et al.,1973; reviewed by Pringle et al.,1981). The "START" event occurs in the late G1 interval of the yeast cell cycle. Although stationary phase cells and mating pheromone arrested cells accumulate in late G1, the rates of total protein and RNA synthesis are different. In stationary phase cells the overall rates of protein and RNA synthesis are dramatically reduced, whereas the rate of protein and RNA synthesis remain unaffected in alpha-factor arrested cells.

v) What happens to TMP1 mRNA levels as cells are permitted to execute the hypothetical "START" event? This question was examined by allowing the G1 arrested cells (stationary phase cells and alpha-factor arrested cells) to resume cell division. As mentioned above both stationary phase and alpha-factor arrested cells accumulate in late G1 at or near "START". Removal of the pheromone alpha-factor or the refeeding of starved cells causes G1 arrested cells to execute "START". After the completion of "START", the cell becomes commited to a complete cell division cycle. I also followed the TMP1

message levels when a cdc4-1 strain was released from stationary phase at the non-permissive temperature (37°C). A culture of cdc4-1 cells released from stationary phase at 37°C will arrest at the CDC4 block during the cell cycle. The CDC4 block follows immediately after the execution of "START" (Hartwell et al.,1973, and reviewed by Pringle at al.,1981).

What mechanism regulates TMP1 gene expression? To answer this question I initially investigated whether cells released from stationary phase required ongoing protein synthesis to induce TMP1 expression. Second, I investigated the effect of increasing the TMP1 gene dosage on the accumulation of TMP1 transcript.

Materials and Methods.

Strains.

The strains used in this study are presented in the table 1. AH22 was also used when it was transformed with plasmids. In this study the name of the transformant will include the parental strain name (AH22) followed by the name of the plasmid. For example, AH22 transformed with pYe(cen3)471 has been designated AH22-pYe(cen3)471.

Plasmids.

All the plasmids used in this study are presented and listed in table 2.

Plasmid constructions.

The plasmid pRS780 (Figure 7, p.56) was constructed using pTL222 (Taylor et al.,1982) and pUC 9. Plasmid pTL222 carried an 830 base-pair PstI-BamHI fragment containing most of the TMP1 gene. Both plasmids pTL222 and pUC9 were digested with the restriction endonucleases PstI and BamHI. The restricted fragments were mixed together and ligated using the E. coli T4 DNA ligase. Restriction endonuclease analysis of pRS780 was carried out as described by Maniatis et al.,1982.

The plasmid pRS(cen3)471 (Figure 7, p.56) was constructed using pYE(cen3)40 (Clark et al. 1982.) and pTL222. Plasmid pTL222 contains the whole TMP1 gene on a

TABLE 1

Strains used

Saccharomyces cerevisiae

Strain	Genetype	Source
AH22	"MATa" can1-1 leu2-3 leu2-112 his4-514	G.R. Fink
314	cdc4-1	Stock Center

Escherichia coli

Strain	Genotype	Source
JF 1754	hsdr lacZ⁻ gal metB leuB hisB436	J.Friesen
JM 101	Δ lacpro supE thi F' traD36 proAB lacI Z M15	Stock Center
MH458	JM 101 recA::Tn10	Johnny Basso

TABLE 2

Plasmids used

Plasmid	Pertinent features	Source
pRS535	pBR322 AMP^R 2μ LEU2 TMP1-lac'ZYA (15.9 Kb)	R.W. Ord (1987)
pRS787	Same (16.1 Kb)	Yip Ho
pRS264i	Same (16.4 Kb)	M.Greenwood
pTL1'	pBR322 AMP^R 2μ LEU2 TMP1 (23.4 Kb)	M.Greenwood (1986)
pYe(cen3)40	pBR322 ARS1 cen3 LEU2 (8.2Kb)	Clark et al. (1980)
pRS(cen3)471	same pYe(cen3)40 TMP1 (10.2 Kb)	This study
pRS(M13)535	HindIII-BglI TMP1-LacZ from pRS535 cloned into M13mp19 (8.7 Kb)	This study
pRS(M13)787	Same. Fragment from pRS787 (8.9 Kb)	This study
pRS(M13)264i	Same. Fragment from pRS264i. (9.3 Kb)	This study
YIP5	pBR322 AMP^R URA3 (5.6 Kb)	Struhl et al. (1979)
M13mp19	lacZ (8 Kb)	Stock center
pUC9	pBR322 AMP^R (3.2 Kb)	Stock center

fragment flanked by Hindlll sites. Plasmid pYe(cen3)40 contains a unique Hindlll restriction site. Both plasmids were digested with Hindlll and the restricted fragments were ligated with the E. coli T4 DNA ligase. Restriction endonuclease analysis of pRS(cen3)471 was carried out as described by Maniatis et al.,1982.

M13 phage derivatives constructions.

The M13 phage derivative pRS(M13)535 (Figure 9, p.63) was constructed using the plasmid pRS535 (R.Ord,1987) and the replicative form (RF) of M13mp19 phage (Messing 1983). Plasmid pRS535 harbors a TMP1-lacZ fusion gene. The Hindlll-Bgll fragment present on pRS535 and indicated in Figure 9, includes all of the TMP1 information and 144 base pairs 5' of lacZ DNA. Both pRS535 and M13mp19 phage were digested with Hindlll and Bgll and the restricted fragments were ligated using the E. coli T4 DNA ligase. Restriction endonuclease analysis of pRS(M13)535 was carried out as described by Maniatis et al.,1982.

The plasmids pRS787 and pRS264i, are identical to pRS535 except for the amount of TMP1 (...) information upstream from the lacZ gene (see Figure 9, p.63). Both plasmids contain a Hindlll-Bgll fragment wich includes the TMP1 sequence and the first 144 base pairs of lacZ DNA. The construction of the M13 phage derivatives pRS(M13)787

and pRS(M13)264i was performed exactly as described above for pRS(M13)535.

Growth media.

All yeast cultures were grown in either rich medium (YEPD), super rich medium (0.1%) or minimal medium (2X YNBD). YEPD medium consists of 2% yeast extracts, 1% bacto peptone and 4% glucose. 0.1% medium consists of 0.1% bacto-peptone, 0.1% yeast extract, 1.34% yeast nitrogen base (without amino acids), 4% glucose and 100µg/ml of the required amino acids. 2X YNBD medium consists of 1.34% yeast nitrogen base (without amino acids) 4% glucose and 100 µg/ml of any required amino acids. AH22 was grown on either YNBD supplemented with 50µg/ml of histidine and leucine, or in YEPD. All AH22 transformants used in this study carry plasmids which contain the LEU2 gene. These transformants were always grown in minimal medium supplemented with histidine. The omission of leucine selects for cells harboring the desired plasmid.

Growth conditions.

All strains, except the temperature sensitive strain ts 314 (cdc4-1) were grown at 30°C in a "walk-in" type incubator. The ratio of the flask to the culture volume was maintained at 5:1. The cultures were always grown with vigorous shaking to ensure adequate aeration.

Strain, ts 314, which contains the cdc4-1 mutation, was either grown at 23°C (permissive temperature) or at 37°C (non permissive temperature) essentially as described above. For the refeeding experiments stationary phase cells were harvested by centrifugation and resuspended in a prewarmed (30°C) liquid medium (to avoid heatshock) to a cell density of $1X10^7$ cells/ml. Samples of cells were harvested and frozen as described in the "RNA isolation". In the cycloheximide experiment, stationary phase cells were resuspended in fresh prewarmed 2X YNBD medium with and without 100µg/ml of cycloheximide.

RNA isolation.

Total yeast RNA was isolated essentially as described by Carlson et al.,1982. Cells samples for RNA isolation were taken when the culture had reached a density of $1X10^7$ cells/ml (mid-log phase) or $1X10^8$ cells/ml (stationary phase). The samples were harvested in the presence of 0.06% sodium azide, and the pellet was either frozen in liquid nitrogen and stored at -70°C, or immediately used for RNA isolation. The pellet was resuspended in presence of 100µl (volume) of glass beads, 100µl of breaking buffer (0.5M NaCl, 0.2M Tris-HCl pH7.5, 0.01M EDTA), and 100 µl of phenol solution < 24:24:1, phenol:chloroform:isoamylalcohol, 1.2mM beta-mercaptoethanol, 0.1% SDS (sodium dodecyl sulfate)>.

The samples were vortexed for 10 minutes then centrifuged at 12,000rpm for 20 minutes. The aqueous phase was transferred to a second tube containing 200 µl of phenol solution, vortexed for 3 minutes and spun down as described above. This step was repeated until no white precipitate was present at the interphase. The aqueous phase was then transferred to a 1.5ml microcentrifuge tube, and was extracted with chloroform at least 3 times (the chloroform phase was removed from the 1.5 ml microcentrifuge tube each time). Finally, the aqueous phase was ethanol precipitated overnight at -70°C with 2.5 volumes of 100% EtOH (ethanol) containing 0.01% diethylpyrocarbonate (DEPC). If the RNA was to be frationated through an oligo-dT cellulose column, the DEPC was omitted because DEPC can damage the oligo-dT column (Bantle et al. 1975). The sample was then centrifuged at 12,000rpm for 10 minutes and the RNA pellet was dissolved in DEPC treated H_2O. DEPC treatment of H_2O was performed by adding DEPC to a final concentration of 0.01%. The solution was then left overnight at room temperature (R.T.). The water was then autoclaved for 90 minutes to get rid of the DEPC. The concentration of the RNA was determined spectrophometrically by measuring the absorbance at 260nm (A 260). The aqueous solution was then kept at -20°C for further analysis.

PolyA$^+$ RNA isolation.

PolyA$^+$ RNA isolation was performed essentially as described by Bantle, 1976. The oligo-dT cellulose column was prepared as follows: approximatively 50 mg of oligo-dT cellulose (from Pharmacia) was mixed with 2 ml of high salt buffer (0.5M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl pH7.5). All the solutions used for the polyA$^+$ isolation have been DEPC treated and autoclaved as previously described. The solution was left at R.T. in the dark for at least 24 hours. The oligo-dT cellulose suspension was then poured in a 2cc syringe containing a small bed of glass wool. The column was washed with 20ml of high salt buffer. PolyA$^+$ RNA fractions were isolated as follows: approximatively 1mg of total RNA was dissolved in 50 µl of low salt buffer (0.01M Tris-HCl pH 7.5, 0.001M EDTA). Once the RNA has been dissolved, 450 µl of dimethylsulfoxide (DMSO) which helps to break down the rRNA-mRNA aggregates, and 50µl of LiCl buffer (1M LiCl, 0.05 EDTA, 2% SDS and 0.01M Tris-Hcl pH 6.5) were added to the RNA solution. The solution was then incubated 5 minutes at 55°C and placed on ice. The RNA solution was diluted in 3ml of cold high salt buffer and applied to the column. The column was washed with high salt buffer until the eluted fractions had an absorbance at 260nm of about zero. The column was filled with an equal volume of low salt buffer and was placed at 45°C for 20 minutes.

The polyA$^+$ RNA was eluted with low salt buffer and 500µl fractions were collected. The concentration of the RNA was determined spectrophotometrically. The polyA+ RNA was either dessicated using a vacuum dessicator, or EtOH precipitated and stored at -70°C.

RNA fractionation by agarose gel electrophorisis.

Total RNA (10 to 20µg), was denatured either with glyoxal or with formaldehyde. Denaturation with glyoxal was performed essentially as described by Thomas 1980. The RNA was fractionated by electrophoresis through a 1% agarose gel in a 0.01M phosphate buffer pH 6.5. The RNA was transferred from the agarose gel to a cellulose membrane (NEN Gene Screen), essentially as described by the manufacturer. Denaturation with formaldehyde was performed as described by Lehrach et al.,1977. The RNA was denatured by incubating at 65°C for 15 minutes in the presence of 50% formamide, 2.2M formaldehyde. The RNA samples were then fractionated on a 0.75% agarose-6% formaldehyde gel. The RNA from the agarose gel was transferred to a nitrocellulose membrane (Gene Screen from NEN) as described by Thomas, 1980. The gene screen membrane was then baked at 90°C for at least 4 hours. The membrane was stored at -20°C.

The RNA blot (baked membrane) was pre-hybridized and hybridized exactly as described by Thomas, 1980. The

membrane was pre-hybridized in 5ml of 99% Formamide, 2ml of 5X P buffer, [1% polyvinylpyrrolidone, 1% ficoll, 0.5% sodium pyrophosphate 5% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 250mM Tris-HCl pH7.5; the 5X P Buffer was filter sterilized], 2ml of 50% Dextran sulfate and 100µg of denatured salmon sperm DNA. The membrane was left to pre-hybridize overnight at 42°C. The RNA was hybridized with 0.5µg of purified DNA fragment, containing either the TMP1 or the URA3 gene, labelled by the nick translation method (Maniatis et al.,1975) using E. coli DNA polymerase 1 (Kornberg fragment). The specific activity of the labelled fragments was usually about $1X10^8$ cpm/µg. 0.5µg of radioactive fragment was denatured at 100°C for 5 minutes, and added to the pre-hybridization buffer. The membrane was then left to hybridize overnight at 42°C. The hybridization solution was removed and the membrane was treated as follows: 1) the membrane was washed twice with 100ml of 2 X SSC (0.3M sodium chloride, 0.03M sodium citrate) at R.T. for 5 minutes with constant agitation, 2) then the membrane was washed twice with 100ml of a solution containing 2 X SSC and 1% SDS at 65°C for 30 minutes with constant agitation, 3) finally the membrane was washed twice with 100ml of 0.1 X SSC at R.T. for 30 minutes with constant agitation. The membrane was placed between 2 sheets of Wattman, #3, to get rid of excess

washing solution. Do not dry the membrane if it has to be probed more than once. The membrane was finally wrapped in a layer of "Saran Wrap" and allowed to expose a CUREX film for 1 or 2 days at -70°C using an intensifier screen (Dupont Co.).

To monitor the denaturation and the quality of the RNA, 5µg of each denatured RNA sample and 5µg of undenatured RNA were also subjected to fractionation by agarose gel electrophoresis. The "sister gel" was photographed after ethidium bromide staining (Maniatis et al.,1975) (see Figure 2A., p.33).

Isolation of DNA fragments.

Most of the RNA blots were probed with purified DNA fragment labelled with ^{32}-P. The TMP1 fragment was isolated from pRS780 (see Figure 7, p.56). This fragment is flanked by restriction endonucleases: Pst1 and Hindlll. The plasmid was digested with Pst1 and Hindlll and the digested fragments were separated on a 3.5% polyacrylamide gel. The elution of the fragment was performed essentially as described by Maxam et al.(1980) with the following modifications. The bands containing the Pst1-Hindlll fragment was cut out of the gel and passed through a 1ml syringe and transferred to a pre-siliconised corex tube. 1ml of elution buffer (500mM ammonium acetate, 10mM Magnesium acetate, 1mM ED . and 1% SDS) was added to

the crushed polyacrylamide. The tube was gently vortexed and incubated overnight in a shaker bath at 37°C. The polyacrylamide was separated from the aqueous solution by centrifugation. The solution was filtered through a 0.45μm nitrocellulose membrane. The filtered solution was extracted with phenol and precipitated in 2 volumes of EtOH. The DNA concentration of the fragment was estimated by gel electrophoresis.

The plasmid YIP5 (Struhl et al.,1979) provided the URA3 fragment for the hybridization analysis. The plasmid carries an 0.88kb PstI-AvaI restriction fragment which contains almost all the URA3 gene. The fragment was isolated as described above.

The plasmid pRS88 was used to probe for the actin RNA. This plasmid contains the whole actin gene (3.8kb).

M13 phage manipulation.

All the M13 phage manipulation were carried out essentially as described by Messing, 1983. The E. coli strain MH458, which is a RecA⁻ derivative of JM101 (generously provided by Johnny Basso) was used for the M13 phage infections. The cells were infected in early exponential phase (OD600 of 0.15) and were allowed to grow with vigorous shaking at 37°C for 4 hours. The isolation of the single stranded M13 phage DNA was carried as described by Messing (1983), with the following

modifications. To avoid contamination of the ssM13 phage with nicked ssM13 phage, the phages suspension was "gently" phenol and chloroform extracted.

Incorporation of ^{35}S-methionine into new proteins.

Stationary phase cells ($1X10^8$ cells/ml) were resuspended in a prewarmed liquid medium supplemented with 5µCi/ml of ^{35}S-methionine and 10µg/ml of cold methionine, as described by Cooper 1977. The culture were incubated with and without 100µg/ml of cycloheximide. 3ml samples were harvested by centrifugation, and the pellets were resuspended in 3ml of 10% ice cold trichloroacetic acid (TCA). The tubes were boiled for 20 minutes to dissociate the radioactive methionine from the tRNA. The samples were then filtered through a 45µm pore membrane and subsequently washed with 15ml of ice cold TCA (10%). The membranes were then dried and placed in 5 ml of scintillation solution . The radiactivity of each membrane was measured in an LKB scintillation counter.

Construction and isolation of the 32mer oligonucleotide primer.

A 15mer oligonucleotide (5' TCCCAGTCACGACGT 3'), complementary to a sequence found in the *E. coli lacZ* structural gene (+36 to +50), was hybridized to sspRS(M13)535. The hybrid was then extended with the *E.*

coli DNA polymerase I (Klenow fragment), in presence of cold dTTP, dCTP, dGTP and radioactive dATP. The reaction was carried out at RT for 15 minutes. 5ul of a 0.5mM cold dATP was added to the solution and the tube was incubated at RT for an additional 15 minutes. The resulting radioactive double stranded fragments were digested with Sau3A (restriction endonuclease) for 2 hours at 37°C. There is a Sau3A restriction site 17 bases 5' to the primer. Digestion with Sau3A therefore generated a double stranded region which will contain a unique 32 base radioactive strand complementary to the unlabelled ssM13 phage hybrid-pRS535. The double stranded extended product was denatured and the single stranded fragments were separated on a denaturing gel. The 32 base fragment was then eluted from the gel as described in the "Isolation of DNA fragments" section of the methods and materials. The final specific activity of the 32mer was $7X10^9$ cpm/µg. The sequence of the 32 mer is:

5' CTAGGGCAGCAAAATGATCCCAGTCACGACGT 3'.

Primer extension.

The single stranded 32 mer primer ($1X10^7$ cpm/1ng), was EtOH-precipitated with 20µg of polyA+ RNA extracted from either AH22 or AH22-pRS535. The precipitates were then resuspended in 10µl of hybridization buffer (100mM NaCl, 20mM Tris-HCl pH 8.3,

0.1mM EDTA). The RNA solution was denatured for 2 minutes at 100°C and immersed in a 45°C water bath for 16 hours. All the above mentioned manipulations were performed in a 1.5 ml microcentrifuge tube sealed in a plastic bag. After 16 hours at 45°C, the solution was diluted in 100µl of reverse transcriptase buffer (50mM Tris-HCl pH 8.3, 100mM NaCl, 10mM MgCl2, 10mM dithiotreitol (DTT) 100µg/ml of actinomycin D, 20µM of each dATP,dTTP,dCTP and dGTP), to which 2.5U of reverse transcriptase were added.The actinomycin was included to inhibit the DNA-DNA polymerase activity of the reverse transcriptase complex. The reaction was carried out at 42°C for 2 hours. The solution was then phenol extracted and ethanol precipitated at -70°C. The DNA was dissolved in 10µl of water, denatured at 100°C for 3 minutes and run on a 8% polyacrylamide-7M Urea denaturing gel.

Dideoxy chain termination method.

ssM13 phage DNA from pRS(M13)535, pRS(M13)787 and pRS(M13)264i were hybridized to the sequencing primer (3'TCCCAGTCACGACGT 5') essentially as described by Sanger et al.,1977. 1µg of ssM13 phage DNA, 20ng of the 15mer, and 1µl of H buffer (100mM Tris-HCl pH 7.9, 600mM NaCl, 66mM MgCl2), were mixed in a screw capped 1.5ml microcentrifuge tube. The tube was sealed in a water proof

plastic bag and immersed in boiling water for 2 minutes. The bag was rapidly transferred to a 65°C water bath to incubate for 15 minutes. The 1.5ml microcentrifuge tube was then left at RT for at least 20 minutes. The hybridized primer was extended using the *E. coli* DNA polymerase 1 (Klenow fragment) in the presence of ddNTP and ^{32}P-dATP (300Ci/mmole). The Klenow reaction was carried out at RT for 15 minutes in a final volume of 5µl. The reaction was stopped by the addition of 2µl of 0.25mM EDTA. The concentration of the ddNTP used in each reaction was calibrated to detect 15bp to 250bp fragments. 1ul of either, 0.25mM ddATP, 0.70mM ddCTP, 0.35mM ddCTP or 0.5mM ddTTP, was added in the appropriate tube. 2µl from each reaction was denatured for 3 minutes at 100°C and immediately loaded on a 8% polyacrylamide-7M Urea denaturing gel. Following electrophoresis the gel was dried at 80°C for at least 1 hour and then was allowed to expose Curex film overnight at RT (no intensifier was used).

RESULTS

SECTION 1: Characterization of the TMP1 transcript.

To initiate this study I wanted to determine; i) whether TMP1 mRNA was polyadenylated, and ii) the size of TMP1 transcript.

TMP1 mRNA is polyadenylated.

Total RNA was extracted from an exponentially growing culture of yeast strain AH22. PolyA$^+$ RNA was isolated using an oligo-dT cellulose column as described in methods and materials. One µg of polyA$^+$ RNA and 10 µg of total RNA were denatured with formaldehyde and then fractionated on a 0.75% agarose-6% formaldehyde agarose gel. The RNA was transferred to a nitrocellulose membrane using the RNA blotting procedure (see materials and methods). The blot was probed for TMP1 mRNA using a radioactively labelled PstI-HindIII restricted fragment isolated from pRS780 (see Figure 7, p52). The PstI-HindIII fragment includes most of the TMP1 structural gene. The autoradiogram resulting from this hybridization is shown in Figure 1A. Densitometric analysis of the 2 lanes revealed that there was approximatively 10 times more TMP1 mRNA in 1µg of polyA$^+$ RNA than in 10 µg of total RNA.

The fractionation of RNA on a 0.75% agarose-6% formaldehyde gel provides conditions where the mobility of RNA molecules (smaller than 2 kilobases) is proportional to their respective molecular weight (Lehrach et al.,1977). Furthermore, the abundance of the rRNA, tRNA, 5S and 4S RNA species within the RNA population (more than 90%), permits visual localization of these species as distinct bands in an RNA agarose gel stained with ethidium bromide (EtBr). The known molecular weights of the rRNA and tRNA species in yeast, as well as the ease with which their mobilities can be determined on agarose gels, permitted the establishment of a standard curve to estimate the size of the TMP1 transcript (Figure 1B). Studies of the standard curve indicated that the labelled PstI-HindIII TMP1 fragment hybridized to a message of approximatively 1.25 kbp in length.

FIGURE 1

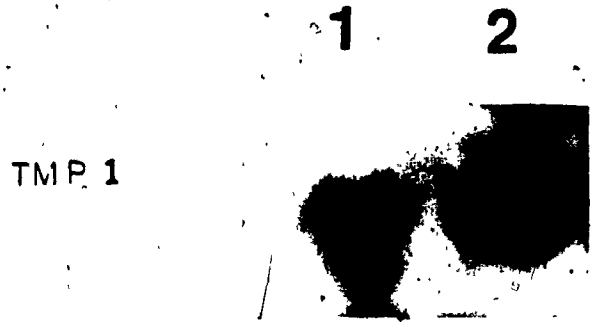
A. Levels of TMP1 mRNA in total RNA and in polyA$^+$ RNA. Total RNA was isolated from a mid-log phase culture of AH22. The polyA$^+$ RNA was extracted from an oligo-dT cellulose column (see methods and materials for details). The RNA was denatured with formaldehyde, fractionated on a 0.75% agarose-6% formaldehyde agarose gel transferred to a introcellulose membrane and probed for TMP1 mRNA using a ^{32}P labelled PstI-BamHI DNA fragment isolated from pRS780 (Figure 7). The specific activity of the labelled fragment was $2X10^7$ cpm/µg. The hybridized membrane exposed X-ray film for 2 days in a Dupont intensifier screen at -70°C. Lane 1) TMP1 specific DNA-mRNA hybrids from 10 µg of total RNA isolated from a mid-log phase culture of AH22. Lane 2) TMP1 specific DNA-mRNA hybrids from 1µg of polyA$^+$ RNA isolated from a mid-log phase culture of AH22.

B. Standard curve for RNA size estimation. The size of 27S, 16S and tRNA were plotted against the distance they migrated in a 0.75% agarose-6% formaldehyde gel. The size of TMP1 mRNA was estimated to be 1.25kb, relative to the standard curve. The upper corner of the panel shows a denatured RNA gel stained with ethidium bromide. 15µg of total RNA isolated from a culture of AH22 were loaded in each lane. The letter"U" indicates that the RNA was undenatured.

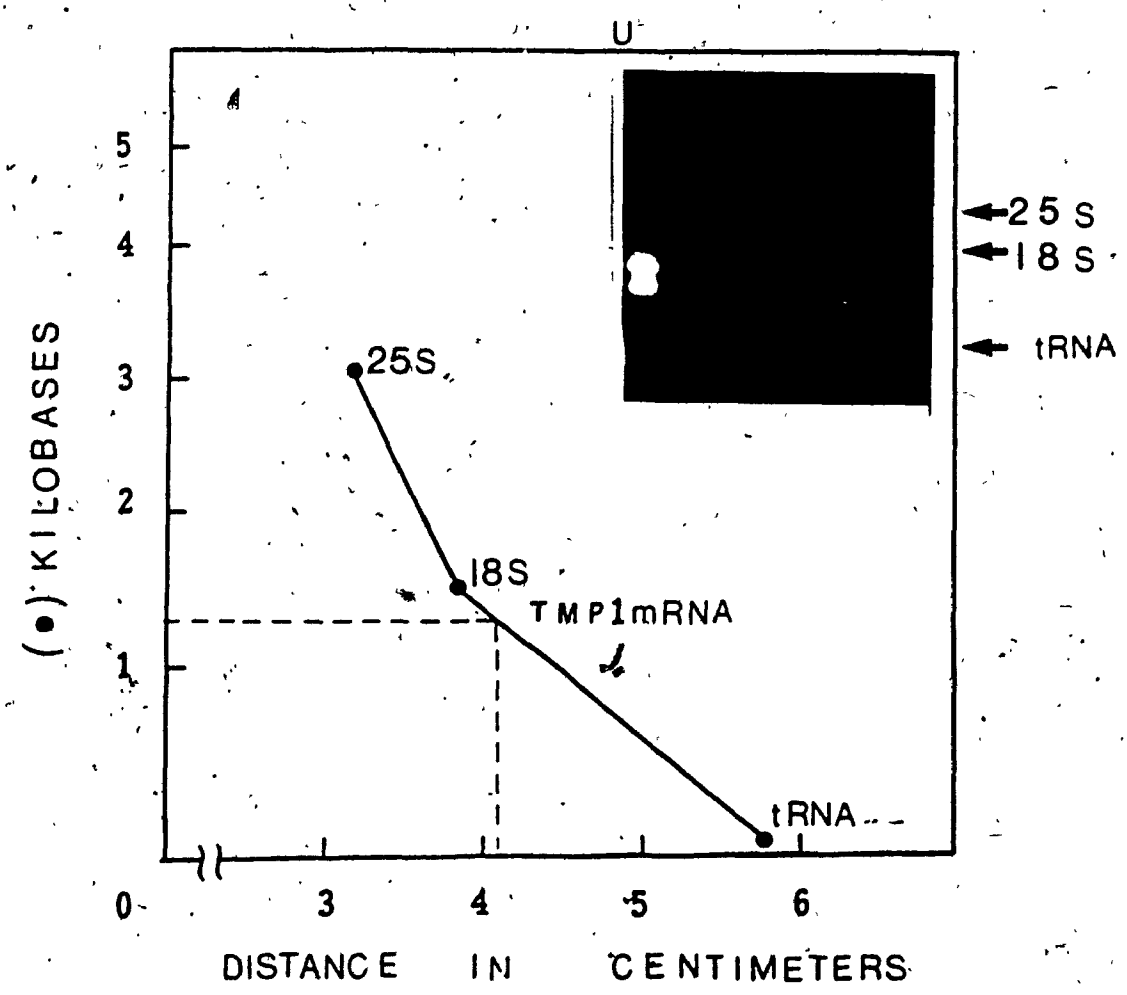
FIGURE 1 cont'ed

The arrows indicate the position of the ribosomal RNA and tRNA species. Note that the 4S and 5S species migrate with the tRNA.

A.


1
2
TMP 1

B.


U
25 S
18 S
tRNA
25S
18S
TMP1mRNA
tRNA
(•) KILOBASES
5
4
3
2
1
0
3
4
5
6
DISTANCE IN CENTIMETERS

SECTION 2: TMP1 mRNA levels present in yeast cells grown under varying growth conditions.

Northern blot analysis was performed to estimate the relative levels of TMP1 mRNA in cultures of AH22 grown in super rich and minimal media, and to follow the levels of TMP1 as a culture of AH22 progressed through the different stages of batch culture growth.

Steady state levels of TMP1 mRNA during logarithmic growth were growth rate dependent.

Previous studies have shown that the level of thymidylate synthase, per yeast cell, increases as the growth rate increases (Greenwood et al.,1986). It was therefore legitimate to reason that the levels of thymidylate synthase mRNA expression might also be growth rate dependent.

The levels of TMP1 mRNA were determined in asynchronous cultures of AH22 grown in super rich and minimal media. The doubling times of a culture of AH22 growing in super rich and minimal media are respectively, 90 minutes and 150 minutes (Greenwood et al.,1986). Total RNA was isolated from an asynchronous mid-log phase culture of AH22 grown in 0.1% (super rich medium) and 2X YNBD (minimal medium). The RNA was then subjected to Northern blot analysis. The RNA blot was successively

probed for TMP1 and URA3 specific transcripts. The autoradiograms resulting from this RNA blot analysis are shown in Figure 2B. Densitometric analysis of the autoradiograms indicated that there was approximatively 6X more TMP1 mRNA in an asynchronous culture of AH22 grown in super rich medium (lane 1), than in an asynchronous culture of AH22 grown in minimal medium (lane 2). In addition, densitometric analysis indicated that the URA3 mRNA levels in cells grown in super rich medium (lane 1) and the levels found in cells grown in minimal medium (lane 2), were not noticably different.

To control for the amount of RNA loaded, 5µg of each RNA sample to be probed for either TMP1 or URA3 specific transcripts, were fractionated on an agarose gel. The gel was photographed after ethidium bromide staining. The stained gel, shown in Figure 2A, indicated that the amount of RNA present in each lane was not noticibly different. Ethidium bromide staining is sensitive enough to distinguish between large, but not small, fluctuations in the amount of RNA loaded.

Levels of TMP1 mRNA decreased when yeast cells were arrested at or near the "START" point of the cell cycle.

Cell division in yeast is regulated by environmental conditions (Hartwell et al.,1973, reviewed in Pringle et

FIGURE 2

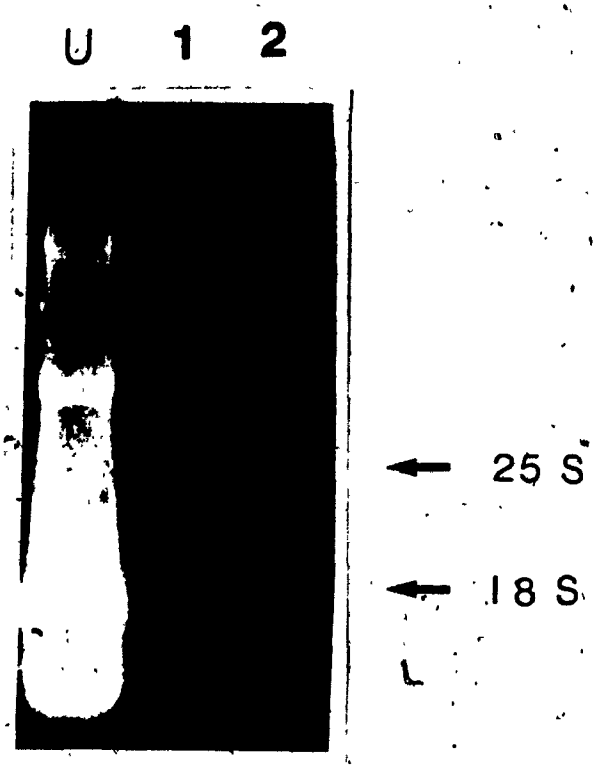
A. Ethidium bromide stained RNA agarose gel. Total RNA was isolated from mid-log phase cultures of AH22 grown in super-rich and minimal media. The RNA was denatured with glyoxal (see materials and methods). A sample (5µg) of the RNA to be probed for both TMP1 and URA3 specific transcripts, was fractionated on an agarose gel and stained with ethidium bromide. The arrows indicate the position of the 27S and the 16S rRNA species. 20µg of undenatured RNA is present in lane "U". Lane 1) 5µg of denatured RNA isolated from a mid-log phase culture of AH22 grown in "super rich" medium. Lane 2) 5µg of denatured RNA isolated from a mid-log phase culture of AH22 grown in minimal medium.

B. RNA blot analysis of TMP1- and URA3 mRNA isolated from cultures of AH22 grown in super rich and minimal media. Total RNA was isolated from cultures of AH22 grown either in super rich or minimal media. The RNA was denatured with glyoxal, and run on a 1% agarose gel. The RNA blot was probed for TMP1 transcript as described in Figure 1. The specific activity of the fragment was $2X10^7$cpm/µg. The hybridized TMP1 specific DNA was then washed from the membrane and the RNA blot was reprobed with 0.5µg of an 0.88kb fragment, isolated from YIP5, which includes most of the URA3 gene. The specific activity of the fragment was $2X10^7$cpm/µg.

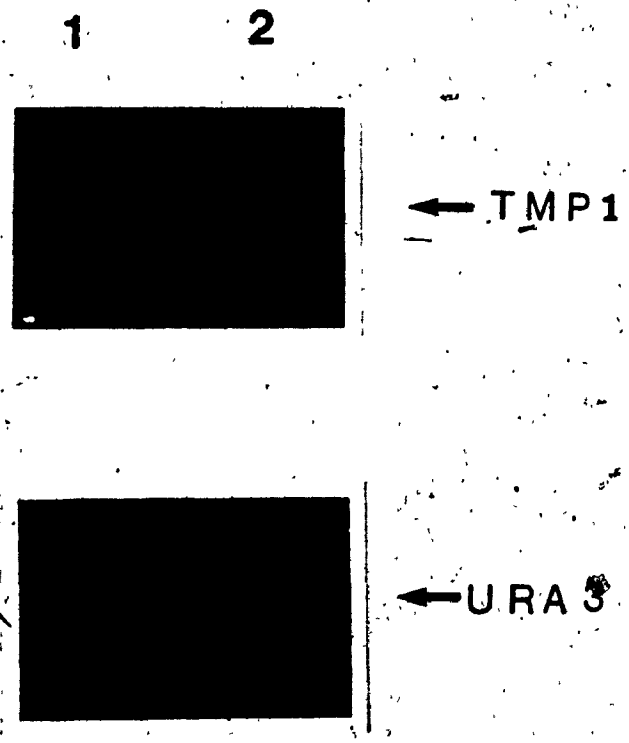
FIGURE 2 cont'd

The membranes were used to expose X-ray films for 2 days in an intensifier at -70°C. Lane 1) Autoradiograms of the TMP1 and URA3 specific DNA hybrids from 15µg of total RNA isolated from a mid-log phase culture of AH22 grown in super rich medium. Lane 2) Same as in 1), with the exception that the RNA was isolated from a culture of AH22 grown in minimal medium.

A.



B.



al.,1981). The execution of a hypothetical "START" event (during late G1) commits the cell to undergo division. Under unfavorable conditions, such as nutrient limitation or exposure of haploid cells to the yeast mating pheromone alpha-factor, yeast cells will not execute "START". I therefore monitored the levels of TMP1 mRNA as yeast cultures were either subjected to nutrient limitation (stationary phase condition), or to the pheromone alpha-factor.

Changes in the levels of TMP1 mRNA as cells entered stationary phase.

Total RNA isolated from a log phase culture of AH22 as it entered stationary phase, was subjected to Northern blot analysis. Visual investigation of the resulting autoradiogram (Figure 3A2) revealed that TMP1 mRNA levels decreased as a log phase culture entered stationary phase. Late stationary phase cells (t=26hours) had no detectable TMP1 mRNA. The RNA blot was reprobed with a URA3 specific probe (Figure 3A3). The autoradiogram revealed that the URA3 mRNA levels also decreased, as the culture of AH22 entered and progressed through stationary phase, to reach undetectable levels at t=26hours. To control for the amount of RNA loaded, 3µg of each RNA sample was run on a "sister gel" and stained with ethidium bromide. The photograph of the stained gel shown

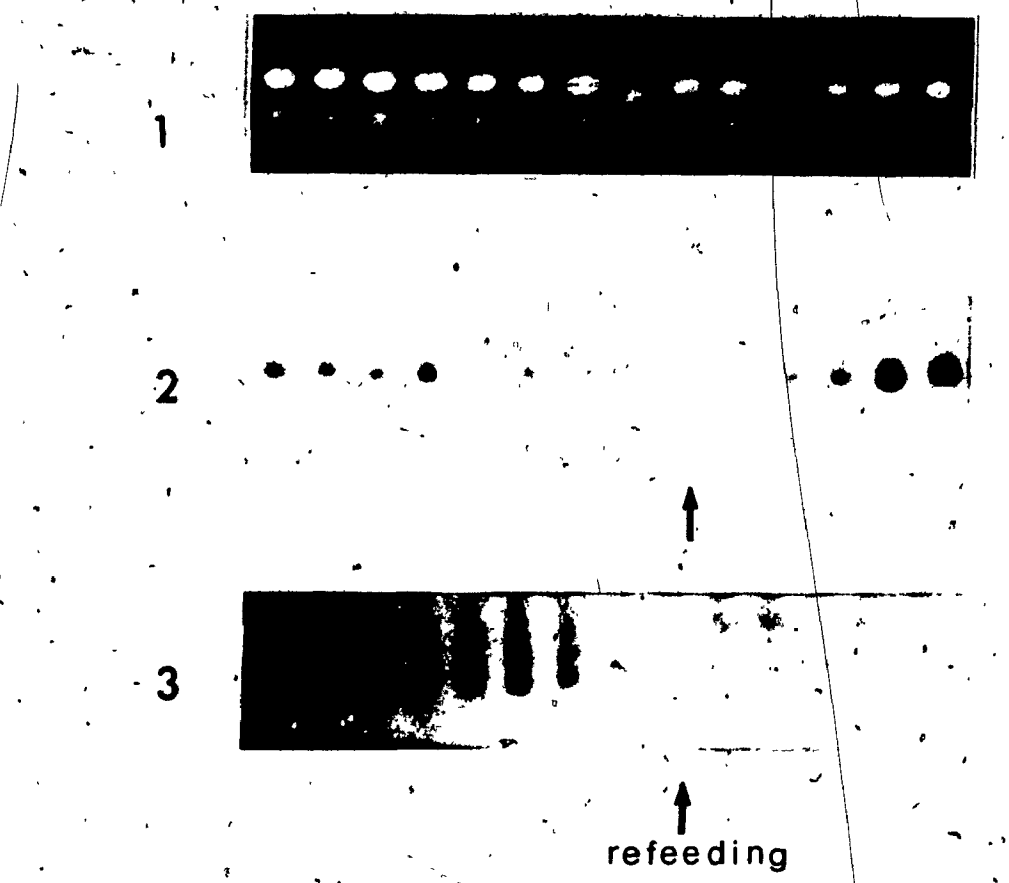
FIGURE 3

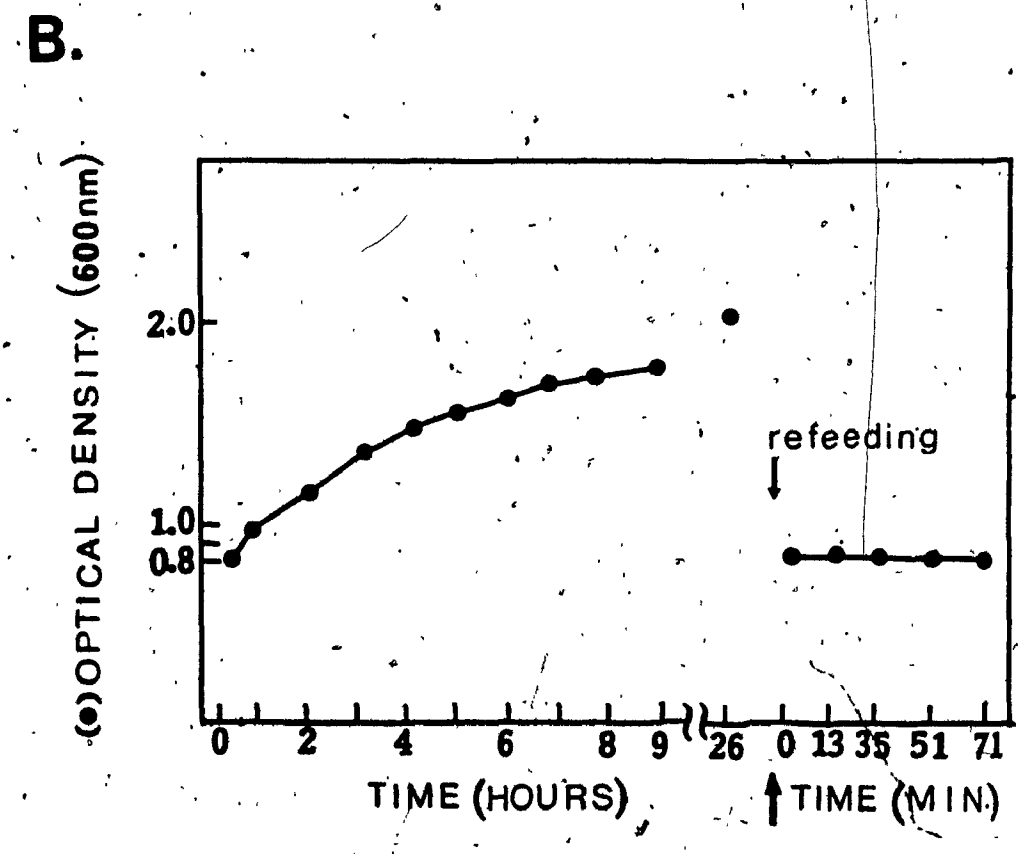
A. Levels of TMP1 mRNA as yeast batch culture entered stationary phase and as it released stationary phase. Cells were grown in minimal medium and samples were harvested at various intervals until late stationary phase was reached. The final sample was taken 26 hours after the start of the experiment. Immediately after the 26 hours sample was harvested, a 10ml sample from the stationary phase culture of AH22 was used to inoculate 100mls of fresh medium. Subsequently, cells samples were harvested at various intervals during growth and were used to quantitate the levels of TMP1 and URA3 transcript. Total RNA was denatured with formaldehyde and was run on a 0.75% agarose-6% formaldehyde gel. Panel A1) Sister RNA agarose gel stained with ethidium bromide. 3µg of denatured RNA isolated at the time points indicated on the X axis in Figure 3B, was loaded in each lane. Panel A2) Autoradiogram of the TMP1 specific DNA-mRNA hybrids detected by RNA blot analysis. Each lane contains 10µg of RNA which was isolated at the times indicated in Figure 3B. The arrow indicates the time when stationary phase cells were resuspended in fresh medium. Panel A3) Autoradiogram of URA3 specific DNA-mRNA hybrids. The TMP1 hybrids were washed from the membrane and the RNA blot was reprobed for URA3 specific RNA as described in 2).

FIGURE 3 cont'd

It should be noted that the bands which appeared above the URA3 mRNA are rRNA-associated bands which are often seen with total RNA blots (Peterson et al.,1985).

B. Growth curve of a culture of AH22 as it entered and progressed into stationary phase and after refeeding with fresh medium. The culture turbidity (A600nm) was monitored as a log-phase culture of AH22 entered stationary phase and after refeeding with fresh medium. It should be noted that the time scale is not linear. The arrow indicates the time when a sample of late stationary phase cells (t=26 hours) were harvested and resuspended in fresh medium.


A.
1
2
3
refeeding


B.
(●)OPTICAL DENSITY (600nm)
2.0
1.0
0.8
refeeding
0 2 4 6 8 9 26 0 13 35 51 71
TIME (HOURS)
TIME (MIN.)

in Figure 3A1, indicated that the amount of RNA loaded in each lane did not fluctuate. The RNA sample from cells harvested at t=13 minutes was misloaded on the sister gel but not on the gel to be probed for the TMP1 transcript.

Changes in TMP1 mRNA levels following mating pheromone induced G1 arrest.

The pheromone alpha-factor arrests progression through the cell cycle of "a" mating type yeast cells at or near "START", an event late in G1 (Bucking et al.,1973). The levels of TMP1 mRNA were monitored as a culture of yeast strain AH22 ("MATa") was exposed to alpha-factor. The autoradiogram resulting from the Northern blot analysis (Figure 4A) revealed that TMP1 mRNA decreased to about 0.3 times the levels present at t=0 by t=40 minutes. As a control, the blot was also probed for the URA3 transcript. The relative levels of TMP1 mRNA, following normalization with URA3 RNA levels, are shown in Figure 4B. The data presented in this figure revealed that the reduced levels of TMP1 mRNA detected at t= 40 minutes, were maintained constant until t=120 minutes. Thereafter, the TMP1 mRNA levels increased dramatically, reaching 4 times the levels observed during logarithmic growth by t=200 minutes (Figure 4A). Such an increase in the levels of TMP1 mRNA can be explained by the culture releasing spontaneously

from alpha-factor arrest near or at t=120 minutes. As the "a" mating type cells metabolize the alpha-factor they will spontaneously release from the alpha-factor induced G1 arrest (Bucking-Throm et al.,1973). As the cells escape arrest they soon proceed through the cell division cycle to S phase; a stage when new buds appear on the mother cells. Previous studies have shown that the appearance of small buds coincides with the onset of S phase (Hartwell et al.,1973). The results presented in Figure 4B, indicated that soon after the levels of TMP1 mRNA increased, the proportion of unbudded cells in the population began to decrease.

The increase of unbudded cells in the population following the exposure to the pheromone alpha-factor (Figure 4B) indicated that the cells accumulated in late G1 in response to the pheromone.

The levels of URA3 and ACTIN RNA were also monitored during the alpha-factor induced arrest. The levels of URA3 and Actin RNA remained constant for 2 and 3 hours respectively, following the addition of alpha-factor (Figure 6). The levels of these two RNA species then decreased significantly reaching respectively 0.3 and 0.2 times the levels observed at t=0.

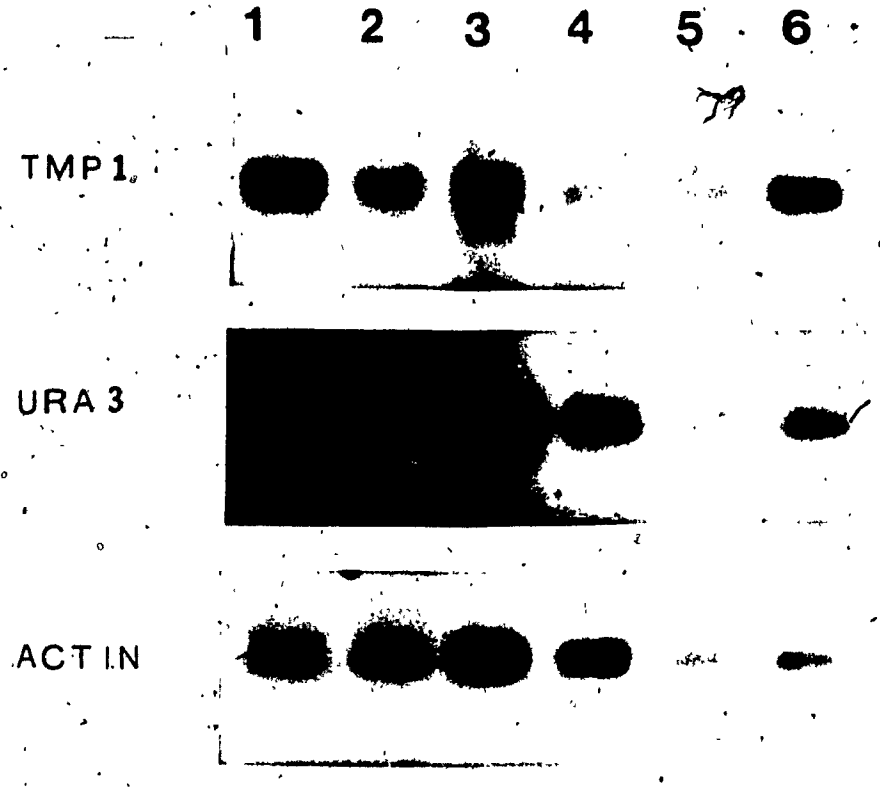
TMP1 mRNA levels following the execution of "START".

FIGURE 4

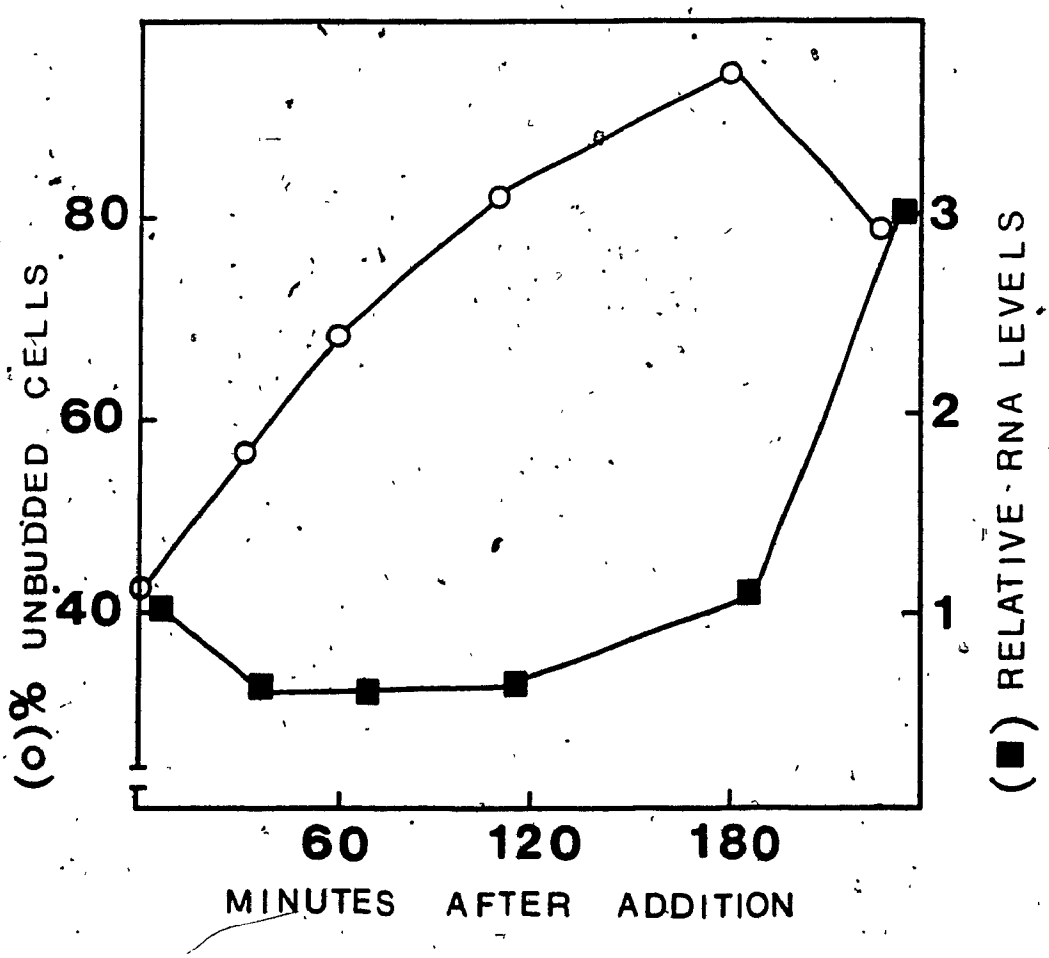
A. Changes in levels of TMP1, URA3 and actin transcripts following the addition of alpha-factor. Total RNA was extracted from cells samples harvested at various time intervals (see panel B) after the addition of alpha-factor. The RNA was denatured with glyoxal, and was probed for TMP1 mRNA as described in methods and materials. The specific activity of the TMP1 probe was $1X10^8$ cpm/µg. The TMP1 specific DNA was washed from the membrane and the RNA blot was successively reprobed for URA3 and actin transcripts. It should be noted that twice as much RNA was loaded onto lane 3. Autoradiograms showing the levels of TMP1, URA3 and actin are presented in A. The time intervals are indicated in panel B.

B. Percentage of unbudded cells and the relative ratios of TMP1 to URA3 mRNA. The relative levels of both TMP1 and URA3 mRNA were determined by densitometric analysis. The ratio of TMP1 to URA3 mRNA (squares), and the percentage of unbudded cells (circles) are depicted (M.Greenwood,1986).

A.


1
2
3
4
5
6
TMP1
URA 3
ACTIN

B.


80
60
40
(o)% UNBUDDED CELLS
3
2
1
(■) RELATIVE-RNA LEVELS
60
120
180
MINUTES AFTER ADDITION

The previous results presented in this study indicated that the levels of TMP1 mRNA present in cells arrested near or at "START" were low relative to the levels in log phase cells. In this part of this study the levels of TMP1 mRNA were monitored as the cells were permitted to execute "START".

TMP1 mRNA levels following the refeeding of a stationary phase culture.

In response to nutrient limitation yeast cells accumulate in late G1 near or at "START" and enter a quiescent state. Quiescent cells rapidly escape G0 to execute "START" and begin proliferatior .f the nutrient limitation is relieved. The levels of TMP1 mRNA were monitored during the transition from a quiescent state to a proliferation state. Cells from a stationary phase culture of AH22 were harvested and resuspended in fresh medium at a final density of $1X10^7$ cells/ml. Total RNA was isolated from cell samples harvested at various times following refeeding (the time is indicated on the X axis in Figure 3B), and used for RNA blot analysis. The levels of TMP1 and URA3 mRNA were determined as described previously. The autoradiogram, shown in Figure 3A2, indicated that the levels of TMP1 transcript increased to detectable levels by t=13 minutes following refeeding. In contrast, the levels of URA3 mRNA remained well below

log phase levels (compare lane t=0 hour with lanes t=0 minute to t=71 minutes, Figure 3A3), for at least 71 minutes following refeeding.

TMP1 mRNA levels in a cdc4-1 mutant

Previous studies have shown that levels of TMP1 mRNA fluctuate periodically during the cell division cycle in yeast (Storms et al.,1984). The levels of TMP1 mRNA peak near the beginning of S phase of the cell cycle. We therefore, wanted to investigate whether the periodic increase of TMP1 mRNA was dependent upon the completion of the CDC4 event. Hartwell et al.(1973) isolated temperature sensitive mutants which arrest their progression through the cell cycle at precise points. Yeast cells carrying the mutation in the CDC4 gene arrest in late G1, shortly after the completion of "START", when grown at the restrictive temperature (37°C).

The mutant strain ts 314, which carries the cdc4-1 allele, was grown to a density of $1X10^8$ cells/ml (stationary phase), at the permissive temperature (23°C). The cells were harvested and divided into 2 equal portions. One portion was resuspended in fresh medium prewarmed to 37°C (non permissive temperature) and the other portion was resuspended in fresh medium at 23°C (permissive temperature). The cultures were

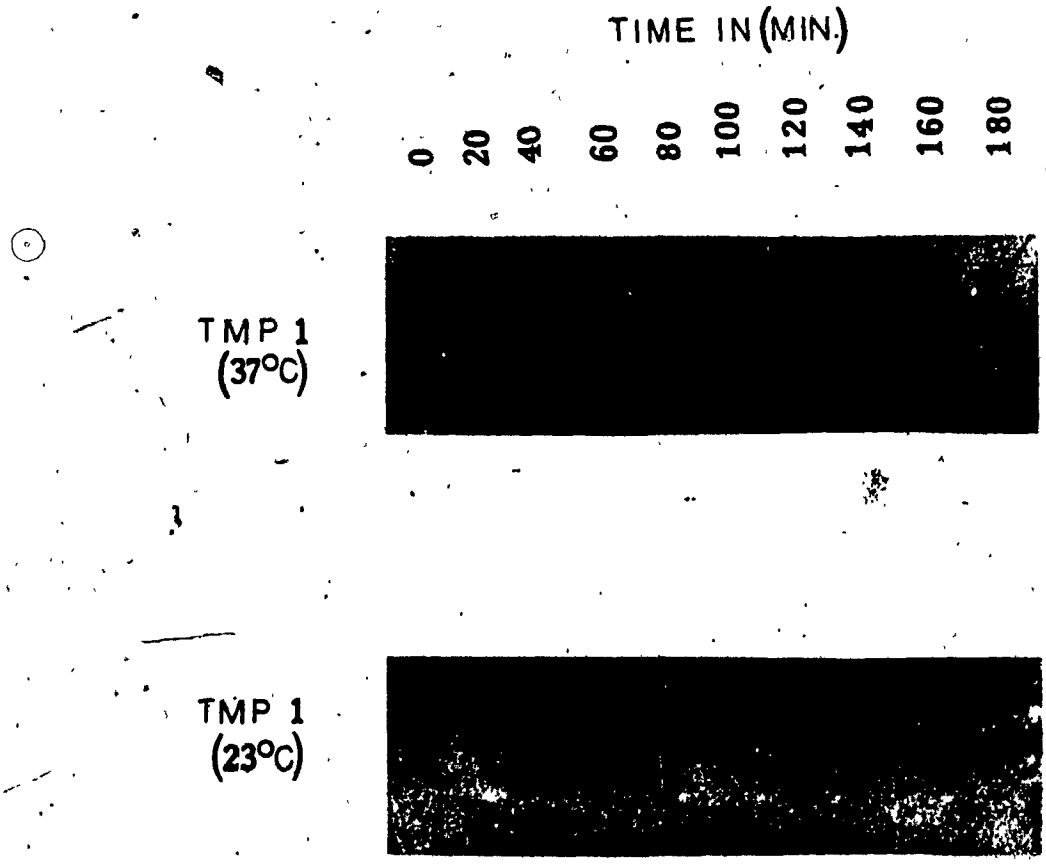
incubated at 37°C and 23°C respectively, and the levels of TMP1 mRNA were monitored at 20 minutes intervals, during the subsequent growth. The autoradiograms resulting from Northern blot analysis of the isolated RNA are shown in Figure 5. The accumulation of TMP1 mRNA in the cdc4-1 population grown at 37°C (non permissive temperature) was detectable at t= 20 minutes after refeeding. Maximum levels were reached at about t=120 minutes. The TMP1 mRNA levels remained high until at least 180 minutes after refeeding. The accumulation of TMP1 mRNA in the cdc4-1 culture grown at 23°C (permissive temperature) was detectable at t= 40 minutes after refeeding of stationary phase cells and remained high until at least t=180 minutes (Figure 5). The pattern of TMP1 transcript accumulation in the culture incubated at the non permissive temperature (37°C), was essentially the same as the pattern of TMP1 mRNA accumulation at the permissive temperature (23°C). These results indicated that the periodic accumulation of the TMP1 transcript did not require the completion of the CDC4 event.

To ascertain that cell cycle specific arrest was obtained with the cdc4-1 strain, I also monitored bud morphology. Hartwell et al.(1973) have shown that as the cdc4-1 cells arrest at the CDC4 block, they start to accumulate multiple small buds (3 to 5 buds/cell). The bud

morphology of a cdc4-1 culture, grown at 37°C (non permissive temperature), showed that over 90% of the cells accumulated multiple buds by t=180 minutes following the temperature shift.

FIGURE 5

A. The effect of cdc4-1 induced cell cycle arrest on TMP1 transcript levels. Two 30ml samples from a culture of stationary phase ts 314 (cdc4-1) cells were harvested and resuspended in prewarmed rich medium (YEPD). The 2 cultures were incubated either at 23°C or 37°C. Total RNA was isolated from samples of cells harvested at the time indicated in panel B. The RNA was denatured with formaldehyde and probed for TMP1 mRNA as described earlier. This figure shows the levels of TMP1 specific DNA-mRNA hybrids at 20 minutes intervals following release from stationary phase. The upper panel follows the levels of TMP1 transcript as the culture released from stationary phase at the non permissive temperature (37°C), and the lower panel when the culture released at the permissive temperature (23°C).


TIME IN (MIN.)
0
20
40
60
80
100
120
140
160
180
TMP 1
(37°C)
TMP 1
(23°C)

SECTION 3: The regulation of TMP1 gene expression.

To investigate the mechanism(s) which regulate TMP1 expression I examined the effect of cycloheximide on TMP1 gene expression following the refeeding of a stationary phase culture. In addition, I investigated the effect of increased TMP1 gene dosage on TMP1 gene expression.

The effect of cycloheximide on TMP1 expression following refeeding of stationary phase cells.

The results presented earlier in this study indicated that the levels of TMP1 mRNA increased to detectable levels within 13 minutes following the refeeding of stationary phase cells (Figure 3A2). This result was remarkable and suggested that T.S. may be one of the first proteins to be synthesized following the refeeding of stationary phase cells. If TMP1 expression represented one of the first events following the refeeding the induction of TMP1 transcription may not require protein synthesis. To investigate whether the induction of TMP1 following the refeeding required protein synthesis, the levels of TMP1 mRNA were monitored as a culture of stationary phase cells released from G0 in the presence of the protein synthesis inhibitor cycloheximide.

Cells from a stationary phase culture of AH22 were

resuspended in fresh medium containing cycloheximide (100µg/ml) and in fresh medium without cycloheximide. Total RNA isolated from samples harvested at various times after refeeding was subjected to Northern blot analysis. The autoradiogram shown in Figure 6A, indicates that the levels of TMP1 transcript increased following refeeding in presence of cycloheximide (addition of cycloheximide at t=0). The accumulation of TMP1 mRNA in presence of cycloheximide was essentially the same as the accumulation observed when the culture was grown without cycloheximide (compare lanes t=0 to t=60 with lane t=10(0) to t=60(0). The numbers in parentheses give the time (in minutes) at which cycloheximide was added to the culture.

Higher levels of TMP1 mRNA were observed when cycloheximide was added at t=(10) and t=(30) minutes following refeeding, as compared to the levels observed when no cycloheximide was added (t=0), or when cycloheximide was added at t=(0).

To show that protein synthesis did not occur in the presence of cycloheximide, I followed the incorporation of ^{35}S-methionine into acid precipitable macromolecules during the refeeding experiment. The results (Figure 6B) showed significant ^{35}S-methionine incorporation detectable by t= 41 minutes following refeeding in the absence of cycloheximide. The incorporation of ^{35}S-methionine

FIGURE 6

A. Levels of TMP1 mRNA following refeeding of a stationary phase culture of AH22 in presence and in absence of cycloheximide. Four samples of cells from a stationary phase culture of AH22 were resuspended in prewarmed fresh medium with or without 100µg/ml of cycloheximide. The first sample of cells were incubated in absence of cycloheximide. Cyclohexamide was added at t=(0) in the second culture, at t=(10) minutes in the third culture, and at t=(30) minutes in the fourth culture. Total RNA was isolated at the indicated time intervals. The RNA was denatured with formaldehyde and probed for TMP1 mRNA as previously described in this study. The autoradiogram presented shows the levels of TMP1 mRNA as a culture of AH22 released from stationary phase in the absence of cycloheximide (-) CHX, or in the presence of 100ug/ml of cycloheximide (+) CHX. The number in parentheses indicates the time, in minutes, when cycloheximide was added to the cultures.

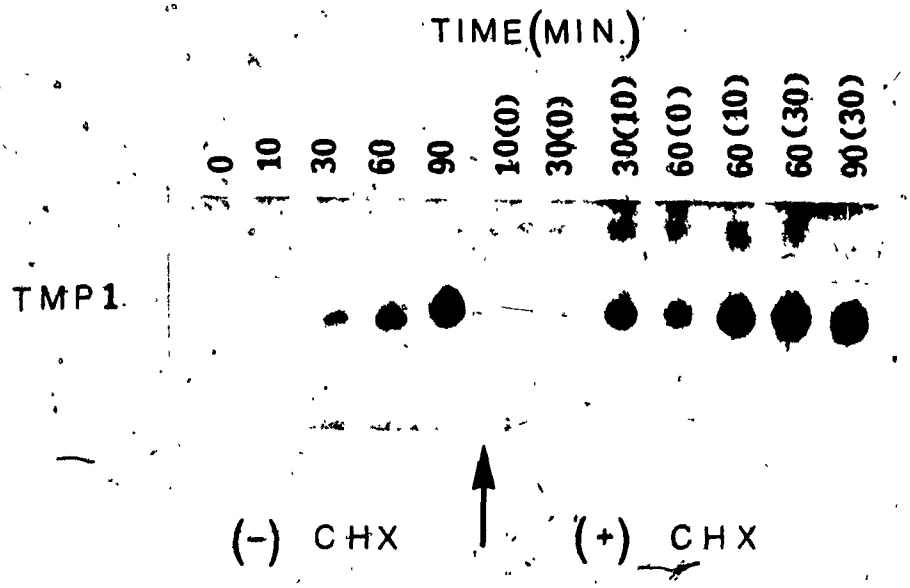
B. ^{35}S- methionine incorporation following refeeding of stationary phase cells in the presence and in the absence of cycloheximide.

Closed circles : ^{35}S- methionine incorporation following release from stationary phase in the presence of 100µg/ml of cycloheximide.

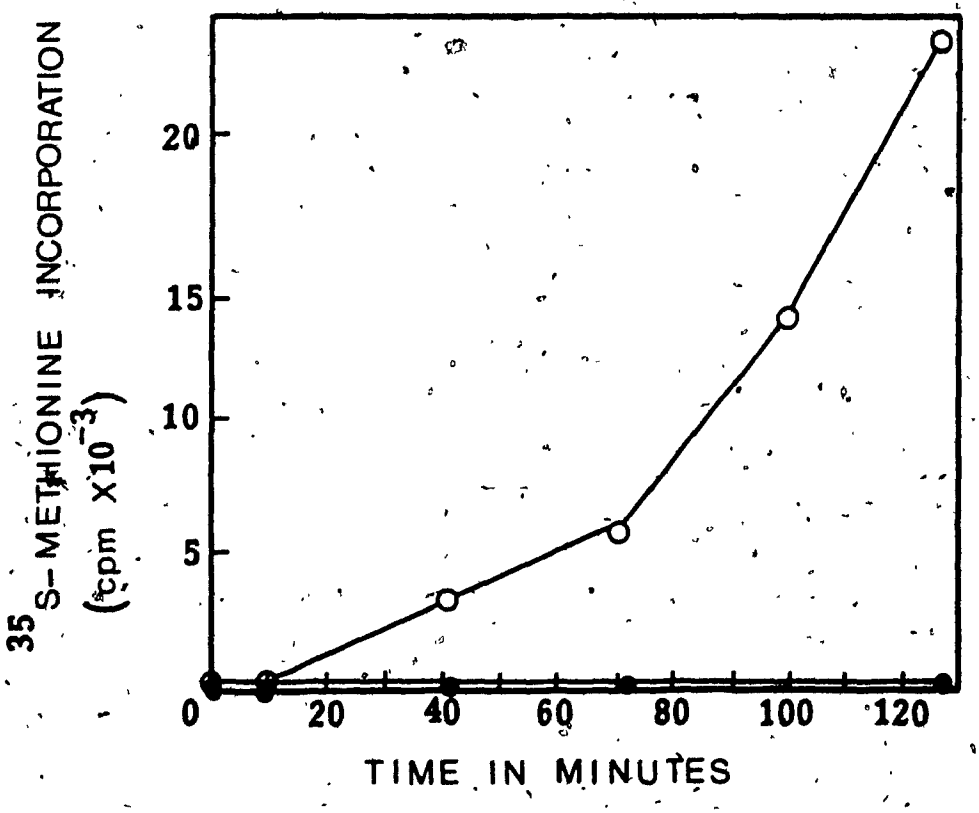
FIGURE 6 cont'd

Open circles : ^{35}S-methionine incorporation following release from stationary phase in the absence of 100µg/ml of cycloheximide.

A.


TIME(MIN.)
0
10
30
60
90
10(0)
30(0)
30(10)
60(0)
60(10)
60(30)
90(30)
TMP1
(-) CHX
(+) CHX

B.


35S-METHIONINE INCORPORATION (cpm X10-3)
0
5
10
15
20
20
40
60
80
100
120
TIME IN MINUTES

increased for at least 120 minutes.

No detectable incorporation of ^{35}S-methionine was observed during the first 120 minutes following refeeding in presence of 100µg/ml of cycloheximide. This indicated that the cycloheximide treatment inhibited protein synthesis in the refeeding experiment.

Gene dosage and TMP1 gene expression.

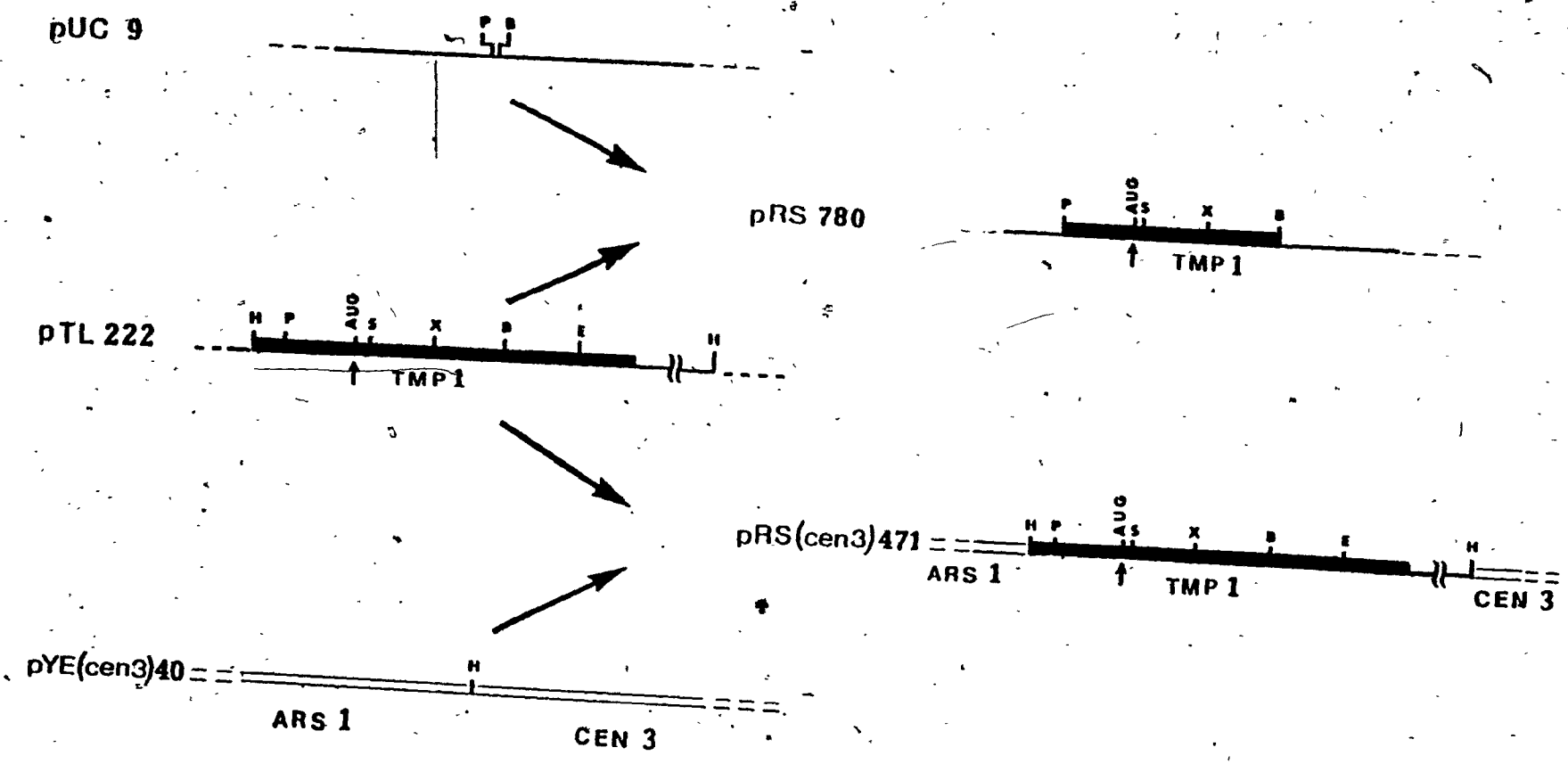
Perturbations which alter the intracellular pools of dTMP has been shown to be lethal to yeast (see Introduction). This might suggest that the levels of dTMP may be precisely regulated by the stringent control of TMP1 expression. To investigate whether yeast compensated for extra copies of the TMP1 gene through transcriptional or posttranscriptional regulatory mechanisms, I monitored TMP1 mRNA levels in AH22 transformants carrying extra copies of the TMP1 gene.

i) Plasmids used in the gene dosage study. The plasmids used in this experiment harbor the entire TMP1 gene. The high copy number plasmid, pTL1', has been described elsewhere (M. Greenwood, 1986). Plasmid pTL1' carries the whole TMP1 gene and a portion of the yeast plasmid 2 micron circle. This type of plasmid is maintained at about 22 to 38 copies/cell (Futcher et al.,1984). Plasmid pRS(cen3)471 contains the whole TMP1

FIGURE 7

Construction of pRS780 and pRS(cen3)471 plasmids. i) Construction of pRS780 plasmid . Plasmids pUC9 and pTL222 were digested with Pstl and BamHl. The restricted fragments were resuspended together and ligated using E.coli T4 DNA ligase. The Pstl-BamHI DNA fragment of pRS780 containing most of the TMP1 gene, was used to detect TMP1 encoded RNA throughout this study.

ii) Construction of pRS(cen3)471 plasmid. Plasmids pTL222 and pYE(cen3)40 were digested with Hindlll. The restricted fragments were mixed together and ligated using E.coli T4 DNA ligase. Restriction endonucleases recognition sites are indicated as follows: P = Pstl, B = BamHl, S = Sau3A,X = Xholl, E = EcoRl, H = Hindlll. 1 centimeter = 0.27 kilobases. The arrow indicates the first ATG. Both recombinants, pRS780 and pRS(cen3)471 are shown in this figure.


pUC 9
P B
pRS 780
P AUG S X B
TMP 1
pTL 222
H P AUG S X B E H
TMP 1
pRS(cen3)471
H P AUG S X B E H
ARS 1
TMP 1
CEN 3
pYE(cen3)40
H
ARS 1
CEN 3

gene, the centromere from yeast chromosome 3 and an autonomous replicating sequence (ARS1). Plasmids like pRS(cen3)471 which harbor yeast centromeres are maintained at between one and two copies per cell (Clark et al.,1980). A detailed description of the plasmid pRS(cen3)471 is presented in the methods and materials section.

ii) The effect of altering TMP1 gene dosage on steady state levels of TMP1 mRNA. AH22 was transformed with the low (pRS(cen3)471) and high (pTL1') copy number plasmids. The steady state levels of TMP1 mRNA were monitored in the parental strain AH22 as well as in the 2 transformants. Since the overall rate of transcription should not be affected by these experimental conditions URA3 mRNA levels were also determined to control for the amount of RNA loaded. The autoradiograms resulting from Northern blot analysis are shown in Figure 8. The relative levels of TMP1 mRNA, after normalization with URA3 RNA, are shown in table 3. The steady state levels of TMP1 mRNA in AH22-pRS(cen3)471 (low copy number,lane 2 Figure 8), and AH22-pTL1' (high copy number, lane 3 Figure 8) were respectively 2X and 40X higher than the levels present in the untransformed parental strain AH22 (lane 1).

FIGURE 8

A. The effect of TMP1 gene dosage on mRNA levels. Total RNA was isolated from a culture of strain AH22 carrying 1 copy of the TMP1 gene per haploid genome (lane 1), from a culture of AH22-pRS(cen3)471 carrying 2-3 copies of the TMP1 gene per haploid genome (lane 2), and, from a culture of AH22-pTL1' carrying approximatively 40 copies of the TMP1 gene per haploid genome (lane 3). The isolated RNA was subjected to RNA blot analysis as described in Figure 1. The RNA membrane was successively probed for TMP1 and URA3 mRNA. The autoradiograms showing the levels of TMP1 and URA3 mRNA are presented in this figure.

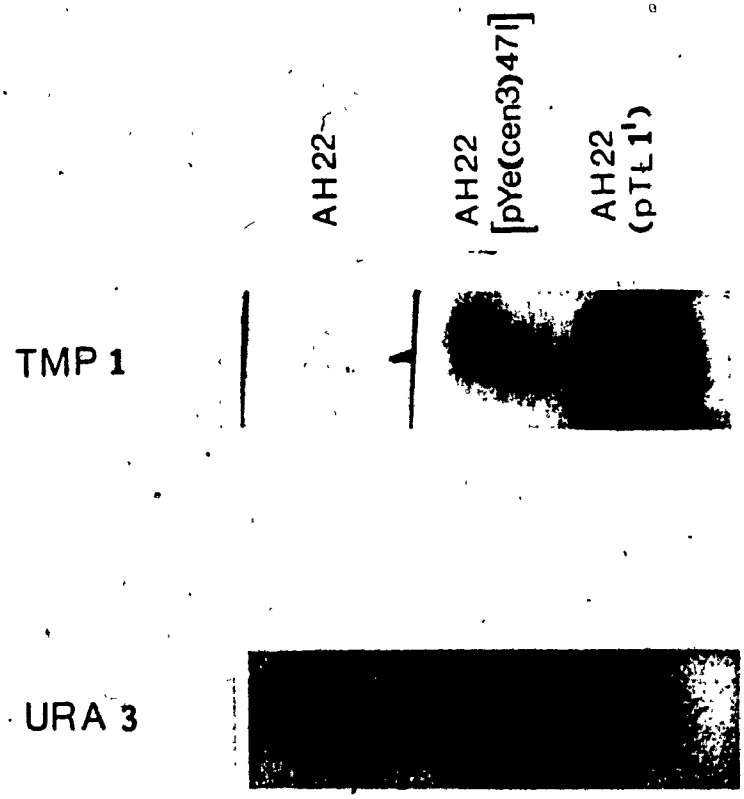

AH22
AH22 [pYe(cen3)47]
AH22 (pTL1)
TMP 1
URA 3

TABLE 3

Table showing the relationship between TMP1 gene dosage, levels of TMP1 mRNA and T.S. activity .

Strains	Copy number[a]	TMP1mRNA / URA3mRNA[b]	T.S.* activity
AH22	1	1	1
AH22 (PYE(CEN3)471)	2-3	2	2
AH22 (pTL1')	20-40	40	10-15

Supercript :

[a]: the numbers represent the number of TMP1 copies per haploid genome.

[b]: the relative levels of TMP1 and URA3 mRNA were determined by densitometric analysis. The numbers presented in this column are the ratio of TMP1 to URA3 mRNA levels.

*: the T.S. activities were provided by M. Greenwood, 1986.

SECTION 4: Characterization of TMP1'-'lacZ hybrid genes.

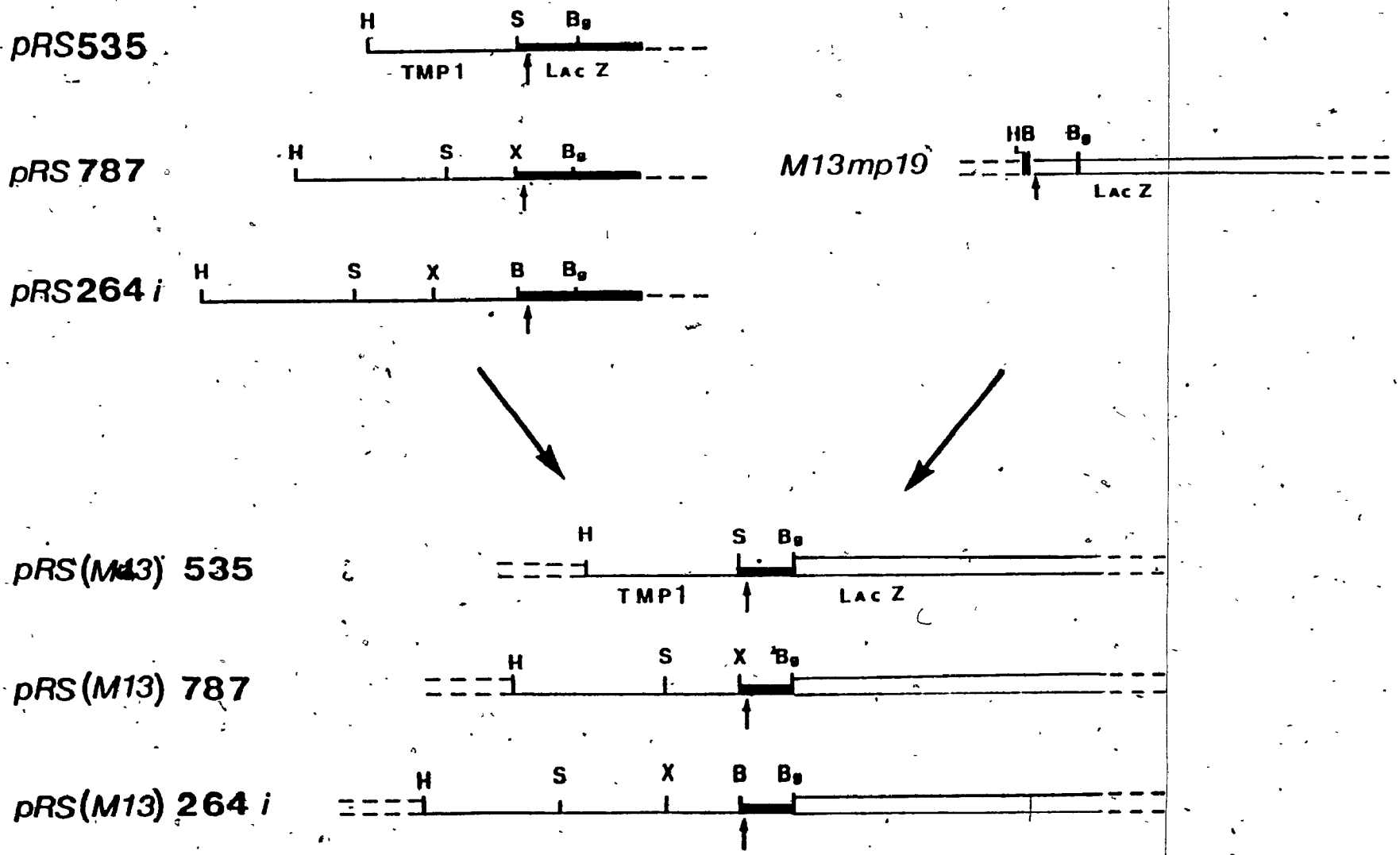
The entire regulatory region plus varying portions of the N-terminal coding region of TMP1 have been fused to the E.coli lacZ gene. The lacZ gene used to construct these fusion genes is missing its transcription and translation start signals as well as the first 8 N-terminal amino acids (see M. Greenwood, 1986 for a detailed description). The levels of Beta-galactosidase expression from these hybrid genes in yeast were shown to fluctuate from one fusion to another (M.Greenwood,1986). To show that these fluctuations were not the results of mutations occuring during the manipulations performed to construct these plasmids, the fusion junction of 3 TMP1'-'lacZ fusion genes were sequenced. Furthermore, the start sites of the TMP1'-'lacZ mRNA were also mapped using the primer extension method to ascertain that the expression of the fusion gene was under the control of the TMP1 promoter region.

Construction of M13 phage derivatives carrying the TMP1'-'lacZ fusion junctions.

The TMP1'-'lacZ fusion genes of pRS535, pRS787, and pRS264i are shown in Figure 9. A detailed description of these plasmids and of their construction have been presented elsewhere (M.Greenwood, 1986 and R.Ord, 1987).

FIGURE 9

Construction of M13mp19 phage derivatives pRS(M13)535, pRS(M13)787, and pRS(M13)264i. Plasmids pRS535, pRS787 and pRS264i were digested with Hindlll and Bglı. The restricted fragments were cloned into the Hindlll-Bglı sites of M13mp19 phage. The recombinant M13 phage derivatives pRS(M13)535, pRS(M13)787, and pRS(M13)264i are shown in this figure. Restriction endonuclease recognition sites are indicated as follows: H =Hindlll, S =Sau3A, X =Xholı, Bgl =Bglı, B =BamHl. The arrow indicated the lacZ region where the 15 mer primer hybridizes. 1 centimeter = 0.183 kilobases.


pRS535
H
S
Bg
TMP1
Lac Z
pRS787
H
S
X
Bg
M13mp19
HB
Bg
Lac Z
pRS264i
H
S
X
B
Bg
pRS(M13) 535
H
S
Bg
TMP1
Lac Z
pRS(M13) 787
H
S
X
Bg
pRS(M13) 264i
H
S
X
B
Bg

These three plasmids are identical except for the TMP1'-'lacZ fusion gene they harbor (Figure 9). The fusion genes differ only with respect to the amount of TMP1 coding information present. All three plasmids have a unique HindIII-Bgl1 restriction fragments which contains the TMP1'-'lacZ fusion junction in addition to a region complementary to the sequencing primer (see materials and methods for the primer DNA sequence). Sequencing analysis required the construction of 3 double stranded M13mp19 phage derivatives carrying the above mentioned HindIII-Bgl1 fragments from either pRS535, pRS787 or pRS264i. The M13mp19 phage derivatives pRS(M13)535, pRS(M13)787, and pRS(M13)264i are shown in Figure 9. The plasmid constructions are described in the materials and methods section.

DNA sequence of the TMP1'-'lacZ fusion junction present on the M13 phage derivatives: pRS(M13)535, pRS(M13)787, and pRS(M13)264i.

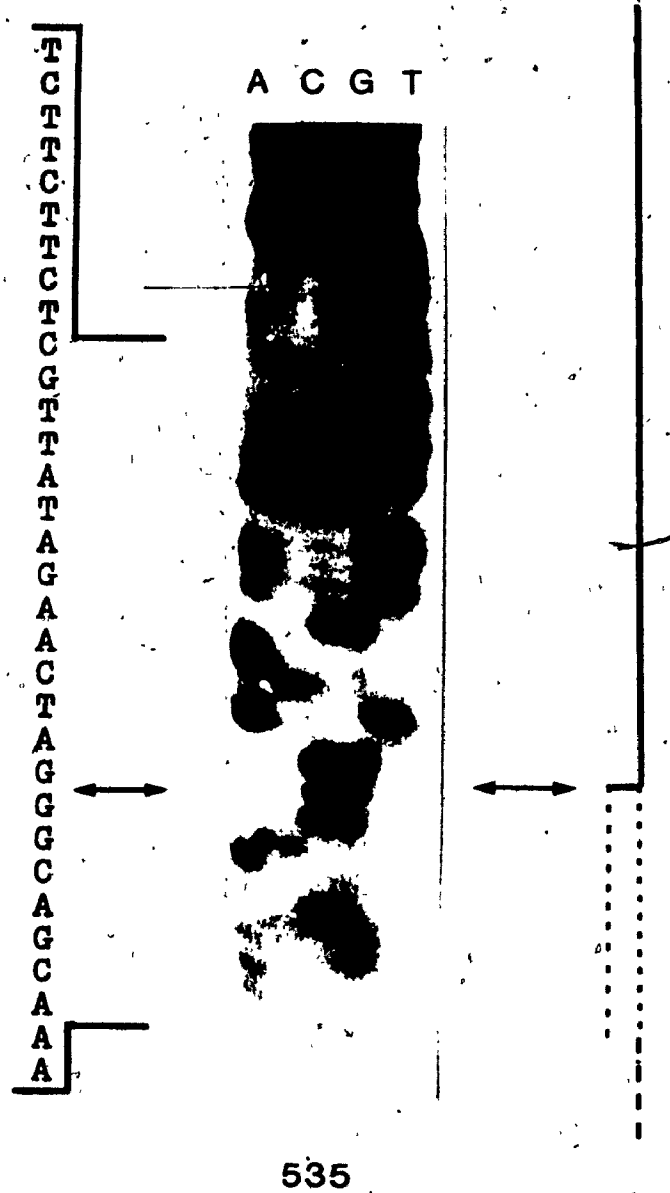
Single stranded M13 phage DNA, isolated from the three M13 phage derivatives carrying the TMP1'-'lacZ fusions, were hybridized to a 15 mer synthetic oligonucleotide (5' TCCCAGTCACGACGT 3') and were sequenced by the dideoxy method (Sanger et al.,1977). The resulting sequence analysis (Figure 10A, 11A, and 12A) indicated that they all have a common GGG sequence at the junction

FIGURE 10

A. DNA sequencing analysis of the pRS535 TMP1'-'lacZ fusion junction. 1µg of single stranded M13 phage derivative pRS(M13)535 was hybridized to 20ng of the 15 mer sequencing primer (described in materials and methods). The primer- M13 hybrid was extended using the E.coli DNA polymerase 1 (Klenow fragment) in presence of ^{32}-P dATP and ddCTP, ddTTP, ddGTP, ddATP dideoxynucleotides (see materials and methods). The extended products were separated by electrophoresis and autoradiographed. Primer extensions of the hybrids are shown in lanes A,C,G, and T. The observed DNA sequence is shown on the left side of the autoradiogram. The sequence relative to lacZ and TMP1 is shown on the right side of the autoradiograph. The arrow indicates the junction between the lacZ and TMP1 DNA sequence in the fusion gene.

B. Expected and observed DNA sequence of pRS535. The expected DNA sequence and the observed DNA sequence are shown.

A.



B.

EXPECTED

TCTTCTTCTCGTTATAGAACTAGGGCAGCAAAprimer

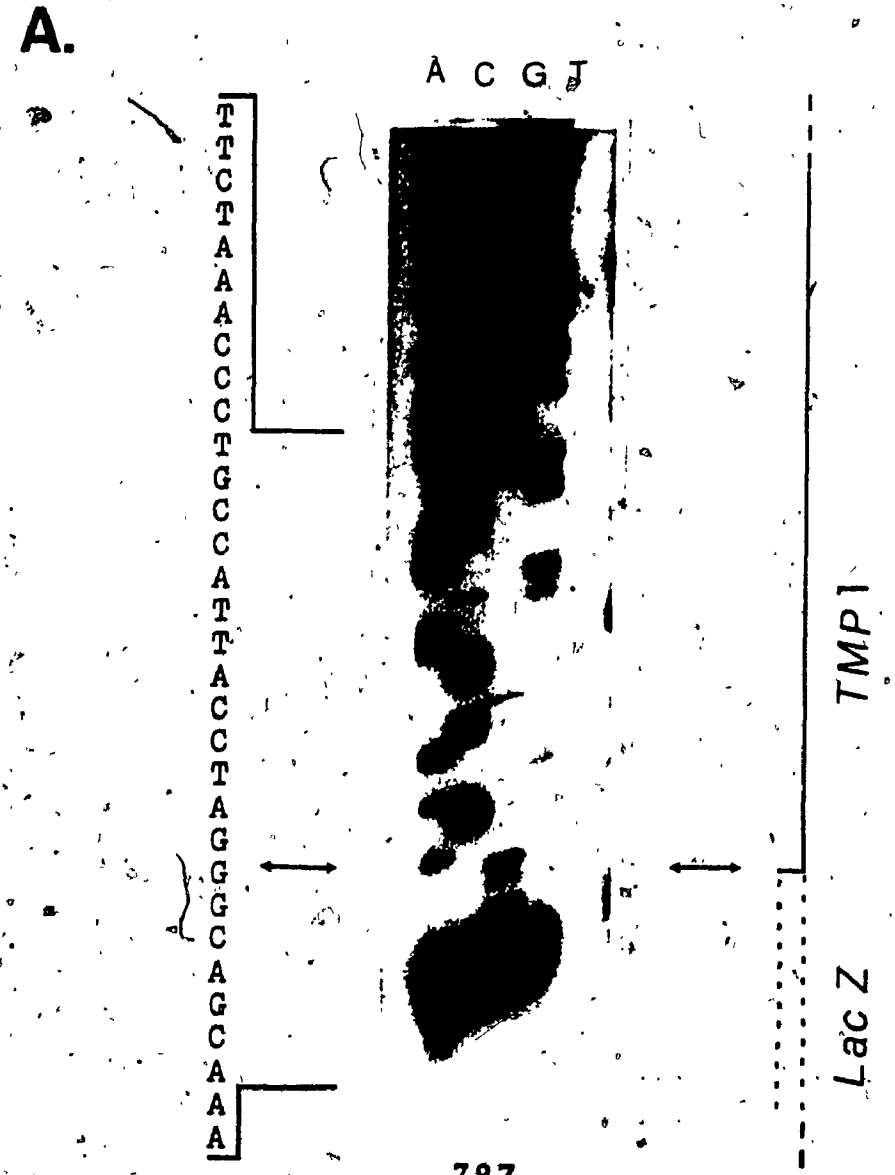
OBSERVED

TCTTCTTCTCGTTATAGAACTAGGGCAGCAAAprimer

FIGURE 11

A. DNA sequencing analysis of pRS787 TMP1'-'lacZ fusion junction. 1µg of single stranded DNA from M13 phage derivative of pRS(M13)787 was hybridized to 20ng of the sequencing primer. The extension was carried exactly as described for pRS535. The extension products are shown in lanes A,C,G, and T. The DNA sequence is shown on the left side of the autoradiogram, and the sequence relative to TMP1 and to lacZ is shown on the right side.

B. Expected and observed DNA sequence of pRS787. The expected DNA sequence and the observed DNA sequence are shown.



B.

EXPECTED

TTCTAAACCCTGCCATTACCTAGGGCAGCAAAprimer

OBSERVED

TTCTAAACCCTGCCATTACCTAGGGCAGCAAAprimer

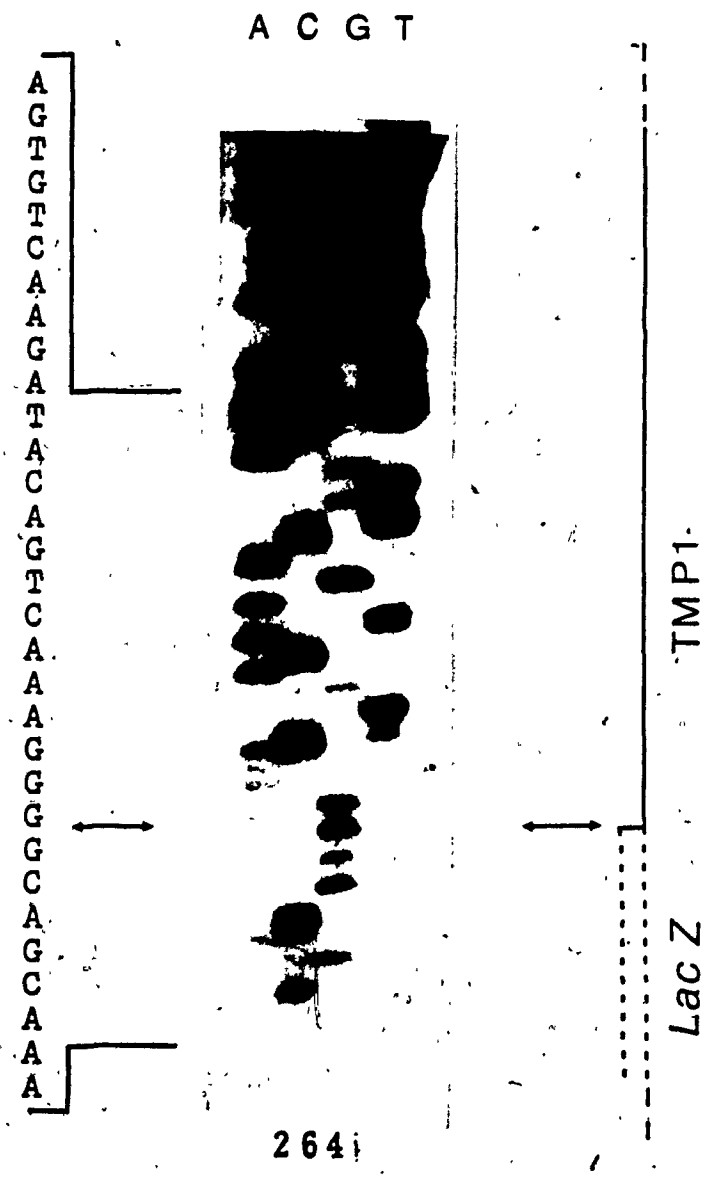
FIGURE 12

A. DNA sequencing analysis of pRS264i TMP1'-'lacZ fusion junction. 1µg of single stranded DNA from M13 phage derivative pRS(M13)264i was hybridized to 20ng of sequencing primer. The extension was carried exactly as described for pRS(M13)535. The extension products are shown in lanes A,C,G, and T.

The DNA sequence is shown on the left side of the autoradiogram and the position of this sequence relative to TMP1 and to lacZ is shown on the right side of the autoradiogram.

B. Expected and observed DNA sequence of pRS264i. The underlined DNA sequence was found to be deleted in the observed DNA sequence.

A.



B.

EXPECTED

AGTGTCAAGATACAGTCAAAGGGGTTTCTTCCTCTTCCT

AGCAGCAAAprimer

OBSERVED

AGTGTCAAGATACAGTCAAAGGGGCAGCAAAprimer

of TMP1'-'lacZ fusion. This was as predicted based on the sequence of the TMP1 and lacZ genes (Taylor et al.,1987 and Kalnins et al.,1983). All the sequenced fusions, except pRS264i, were identical to the predicted TMP1'-'lacZ fusion sequences based on the methods used for their construction (see Figure 10B and 11B). The DNA sequence analysis of pRS264i revealed that 21 nucleotides were deleted during plasmid's construction (Figure 12B).

Mapping the transcription start sites of the TMP1'-'lacZ fusion gene on plasmid pRS535.

To ascertain that the transcription of the TMP1'-'lacZ fusion genes were under the control of the TMP1 promoter region, the 5' end of pRS535 mRNA was mapped by primer extension.

First a single stranded 32 mer oligonucleotide, complementary to the lacZ portion of pRS535 mRNA, was synthesized (details of this synthesis are described in materials and methods). The labelled 32 mer was hybridized to 20 µg of polyA$^+$ RNA extracted from AH22 transformed with pRS535 and the hybridized primer was extended with reverse transcriptase. To control for nonspecific hybridization of the primer to yeast RNA, primer extension was also performed using 20 µg of polyA$^+$ RNA extracted from AH22.

The primer extension products and a DNA sequencing

ladder are shown in Figure 13A. Lane 1 shows the band pattern obtained from the primer extension of the 32 mer using RNA isolated from AH22-pRS535. The multiplicity of the bands indicates heterogeneity of the 5' ends in the TMP1'-'lacZ mRNA population. The specificity of these bands to TMP1'-'lacZ mRNA was confirmed by the absence of any detectable primer extended product in the untransformed parental strain AH22 (lane 2 Figure 13A).

To precisely determine the size and the start sites of the extended fragments within the TMP1 promoter region, pRS(M13)535 was sequenced by the dideoxy method and was simultaneously run with the primer extended products. The location of the 10 TMP1'-'lacZ transcriptional starts sites detected by this analysis are shown in Figure 13B.

FIGURE 13

A. Mapping the 5' termini of the TMP1'-'lacZ mRNA expressed from pRS535 in yeast, by primer extension. 20µg of polyA$^+$ RNA isolated from a mid-log phase culture of AH22 either untransformed or transformed with pRS535, was hybridized to a ^{32}P labelled 32mer primer ($2.5X10^6$ cpm). The RNA-primer hybrids were extended with 1U of reverse transcriptase (see materials and methods). The extension products were denatured and run on a 8% polyacrylamide-7M UREA denaturing gel. The gel was dried at 80°C for 1 hour and then allowed to expose Curex film overnight at R.T. (no intensifier was used.). 1) Primer extension products detected in 20µg of polyA$^+$ RNA isolated from AH22-pRS535 transformant. 2) Primer extension products detected in 20µg of polyA$^+$ RNA isolated from a culture of untransformed AH22 strain (no extension product was detected in lane 2). The C,G, and T lanes contain the products of dideoxy sequencing reaction using the M13 phage derivative pRS(M13)535 as template and the 15 mer sequencing primer (see materials and methods). The DNA sequence of the C,G,and T ladder is shown adjacent to the autoradiogram. The A reaction is not shown.

FIGURE 13 cont'd

B. Diagram depicting the location of the TMP1'-'lacZ mRNA 5' termini relative to the TMP1 DNA sequence. The DNA sequence of the TMP1 gene between base pair +14 and -114 is shown in this figure. The potential TATA boxes are underlined. The start codon of TMP1 coding region is shown with broken line. The black circles indicate the sites of transcription initiation.

A.

535
C G T
1
2
CGTTTC
GCTTTC
TGTTTT
GCTTTC
CTGCCC
TTGTTT
GTTTTT
TCTGT
TCCTC
TCTCG
GTTTTG
TCTTTG
CCTGT
CCTCT
CTGTC
CTGCC
TTTTT

B.

-113
3' AAATGATATCGTATCGACAATTAACATAAAAGATTTGAACATTCACTGACACATATAGTTTGTTATTT
-100
-80
-60
-40
-20
1
CATAGTTCCTCTCTCGAAGTATTGTCTTGCCATGTCCTACTGATATACCTGCCTTTTTT 5'

DISCUSSION

SECTION 1: Characterization of the TMP1 transcript.

The major findings discussed in this section are: i) TMP1 mRNA was polyadenylated. ii) The TMP1 gene had at least 10 transcriptional start sites.

TMP1 mRNA was polyadenylated.

Total RNA was fractionated on an oligo-dT cellulose column, and the polyA$^+$ RNA fractions were analysed by Northern blot analysis. The results presented in Figure 1A, revealed that 1ug of polyA$^+$ RNA contained about 10 times more TMP1 transcript than 10ug of total RNA. The increased levels of TMP1 transcript in the polyA+ fraction clearly showed that the vast majority of the TMP1 transcripts were polyadenylated.

Most eukaryotic mRNA species contain a polyadenylic acid sequence at their 3' end. Although, a small fraction of the mRNA in higher eukaryote is not polyadenylated (i.e. histone mRNA, Adesnik et al.,1972), all yeast mRNA species studied to date possess a sequence of polyadenylic acid at their 3' end.

The TMP1 gene had multiple transcriptional start sites

The TMP1 transcriptional start sites were mapped

by primer extension using polyA$^+$ RNA isolated from the yeast strain AH22-pRS535. The results shown in Figure 13A, indicated that the TMP1'-'lacZ mRNA had at least 10 detectable transcriptional start sites located between two TATA like sequences (at position -60 and -109), and the first translated codon (position +1).

The presence of multiple transcriptional start sites is a common features of many yeast genes including: HIS 3 (Hinnebuch et al.,1983), MAT (Nasmyth et al.,1984), URA3 (Rose et al.,1983), and CYC1 (Faye,1981).

The laws governing the initiation of mRNA transcription in yeast are not completely understood. However, recent studies on the initiation of transcription in yeast have revealed that "upstream activating sequences" (UAS), TATA elements, and sequences immediately adjacent to each transcriptional start site, are sufficient to specify the accurate initiation of transcription. These studies have shown that deletions or point mutations in the " TATA " sequence reduces the efficiency of transcription and alters the initiation sites (McNeil et al.,1986). Similarly a mutation in the vicinity of a transcriptional start site (1 to 5bp, either upstream or downstream of the initiation site) can result in the inacurrate initiation of transcription (McNeil et al.,1985). The transcriptional start sites used by the pRS535 encoded gene are essentially identical to those

used by the *TMP1* gene itself (E. McIntosh personal communication). Altogether, these observations suggested that the *TMP1* sequence fused to *lacZ* in plasmid pRS535 contained the DNA information necessary to accurately initiate transcription.

SECTION 2: TMP1 gene expression under various growth conditions.

The levels of TMP1 mRNA were monitored as the yeast strain AH22 was grown in different media and as it progressed through the different stages of batch culture growth. The major findings were as follows: i) The steady state levels of the TMP1 mRNA in log phase cultures were growth rate dependent, ii) TMP1 mRNA levels decreased as batch cultures entered stationary phase, iii) TMP1 mRNA levels decreased following the addition of alpha-factor to an asynchronous culture of "MAT a" cells, iv) TMP1 mRNA levels increased dramatically immediately after refeeding of stationary phase cells, and v) CDC4 gene product was not required to induce TMP1 gene expression.

Steady state levels of TMP1 mRNA were growth rate dependent.

TMP1 mRNA levels were monitored in cells growing in super rich and minimal media. The results showed that TMP1 mRNA levels increased as the growth rate increased (Figure 2). The growth rate dependent accumulation of TMP1 mRNA levels in an asynchronously growing culture can be explained by the periodic transcription of the TMP1 gene during the cell cycle.

TMP1 mRNA levels fluctuate in a cell-cycle

dependent fashion. Message levels peak during late G1 after the completion of "START", near the beginning of S phase (Storms et al.,1984). Furthermore, in yeast (Johnston et al.,1977), as well as in mammalian cells (Pardee et al.,1978), the increase in doubling time is accompanied by an increase in the time that the cell spends in the G1 phase before "START". The S, G2 and M cell cycle intervals remain unaffected by changes in the growth rate of yeast (Hartwell et al.,1977, reviewed in Pringle et al.,1981). Therefore the accumulation of TMP1 mRNA as each cell in an asynchronous population traverses the interval between "START" and the onset of S phase should be unaffected by changes in the generation time. However, the levels of TMP1 mRNA in an asynchronous culture should be inversely proportional to the length of the cell cycle, since the proportion of cells being in the G1 phase before the completion of "START", increases proportionally as the generation time increases.

TMP1 mRNA levels decreased as cultures entered stationary phase.

Since TMP1 mRNA levels were growth rate dependent (discussed in the preceeding section), we predicted that TMP1 message levels would decrease as a batch culture entered the stationary phase. To test this prediction TMP1 mRNA levels were monitored as a batch culture of

yeast strain AH22 entered stationary phase. The decrease in the TMP1 mRNA levels observed in early stationary phase and as the cells progressed into GØ, could therefore be explained by the increased number of cells accumulated in late G1 prior to "START", a cell cycle stage when TMP1 mRNA levels are low. The autoradiogram presented in Figure 3A, indicated that the levels of URA3 and TMP1 transcripts decreased as the culture entered stationary phase. Studies in our lab, have shown that the levels of URA3 mRNA do not fluctuate during the cell cycle in yeast (Storms et al.,1984). Therefore, although both TMP1 and URA3 mRNA levels decreased in a similar fashion during the experiment, the decrease in the URA3 RNA levels need not be explained by periodic gene transcription. Previous studies have shown that as yeast cells enter and progress through stationary phase, total RNA synthesis decreases to non-detectable levels (Boucherie H.,1985). These observations suggested that the decrease in URA3 mRNA levels might be the result of a decrease in the overall rate of RNA synthesis during stationary phase. Similarly, the decreased rate of RNA synthesis occuring during stationary phase, might also explain the reduced levels of TMP1 mRNA in stationary phase cells.

Steady state levels of TMP1 mRNA decreased during alpha-factor induced arrest.

Stationary phase cells accumulate in late G1 of the cell cycle near or at "START". Although the decrease observed in TMP1 mRNA levels in stationary phase culture was believed to be the result of an increased number of cells being in the G1 phase, a period during the cell cycle where TMP1 mRNA levels are reduced (Storms et al.,1984) we may not rule out that the observed decrease was the result of metabolic events specific to stationary phase (i.e. such as an overall decrease in the rate of RNA synthesis). To ascertain whether yeast cells arrested at or near the "START" point of the cell cycle had reduced levels of TMP1 mRNA, I monitored the TMP1 mRNA levels at various time following the addition of alpha-factor to an asynchronously growing culture. The population of cells in an alpha-factor arrested culture, like a stationary phase culture, accumulates in G1 phase prior to or at "START".

The results presented in Figure 3, showed that TMP1 mRNA levels, relative to URA3 mRNA levels, decreased when an asynchronous culture was arrested with the mating pheromone alpha-factor. These results showed that cells arrested in G1 prior to or at "START" have reduced levels of TMP1 mRNA relative to the cells of an asynchronous population. This analysis also indicated that after a prolonged period following the addition of alpha-factor (t=180 minutes), TMP1 mRNA levels increased

dramatically reaching levels about 4 fold greater than the steady state levels observed in an asynchronously growing culture (Figure 4A, lane 1). The periodic increase in the TMP1 mRNA levels as well as the synchronization of the population by alpha factor could explain this dramatic increase in the levels of TMP1 message. As cells in the arrested population spontaneously released from the effect of alpha-factor, they executed "START" and progressed through the cell cycle as a synchronised culture. Therefore all the cells in the population induced TMP1 gene expression in a synchronous fashion as they reached the onset of S phase.

Similarly, studies on two other yeast genes which are regulated genes involved in chromatin replication, CDC9 and H2B , have shown that the levels of these 2 messages decrease during alpha-factor induced G1 arrest (Peterson et al.,1985; and Hereford et al.,1982).

Altogether these results suggest that: i) the expression of the TMP1 gene was turned down during the G1 phase prior to "START", and ii) completion of the alpha-factor sensitive step "START", was required for the induction of TMP1 expression.

TMP1 mRNA accumulated early after refeeding of stationary phase cells .

TMP1 mRNA levels increased to detectable levels

within the first 13 minutes following the refeeding of stationary phase cells. Furthermore, TMP1 mRNA levels continued to increase reaching levels 5 time greater than the levels found in exponential phase cells (see Figure 3). Meanwhile, the levels of URA3 transcript remained undetectable for at least 90 minutes following the refeeding of stationary phase cells. These results suggest that: i) the increase in the TMP1 mRNA levels occured almost immediately after the refeeding of stationary phase cells, and ii) two distinct mechanisms governed the expression of the TMP1 and the URA3 genes.

TMP1 expression was induced before the CDC4 mediated cell cycle event.

The rapid accumulation of TMP1 mRNA as cells released from stationary phase suggested that the transcription of TMP1 might have occured very early in the cell cycle, either at or near "START". The levels of TMP1 mRNA were therefore monitored in a temperature sensitive cell division cycle mutant (cdc4-1). This mutant when grown at the non-permissive temperature (37°C), blocks its progression through the cell cycle just after completion of the "START" event (Hartwell et al.,1973). The results presented in this study (see Figure 5) showed that TMP1 mRNA levels increased as a cdc4-1 culture was released from stationary phase at the

non-permissive temperature (37°C). This suggested that: i) the periodic increase in the levels of TMP1 mRNA took place at a point prior to or concomitant with the CDC4 block, and ii) the CDC4 gene product was not required for TMP1 gene expression.

Protein synthesis is required for the initiation of DNA replication but not for the completion of DNA synthesis once DNA synthesis has been initiated (Hereford et al.,1974). Furthermore, cells synchronized with alpha-factor or cells arrested at the CDC4 block cannot initiate DNA replication if cycloheximide is added as alpha factor is removed or as the cdc4 cells are allowed to grow at the permissive temperature. This suggests that protein synthesis, required for chromatin replication, is not completed prior to the CDC4 mediated step. When these results and the results discussed above were taken together they indicated that TMP1 gene expression occured early in the cell cycle at a time when not all the proteins required for DNA replication have been synthesized.

The expression of the yeast genes CDC9 and H2B, which code for 2 proteins required during S phase (DNA ligase and histone 2B respectively), occurs between "START" and S phase (Peterson et al.,1985; Hereford et al.,1982). Recent studies, presented at the 13th International Yeast Meeting, 1986, showed that induction

of CDC9 and CDC8 occurs before the CDC4 mediated event during the cell cycle. These observations suggested that these genes and TMP1 might be expressed with similar "timing" during the cell cycle and therefore might share the same regulatory mechanism(s) for controlling the "timing" of their expression during the cell cycle.

Altogether, these results suggested that the cell cycle dependent induction of TMP1 took place soon after the execution "START" but prior to or at the CDC4 dependent step in late G1.

SECTION 3: Regulation of the TMP1 gene expression.

In this part, I discussed some of the regulatory mechanisms which govern the periodic expression of TMP1. The main conclusions drawn were: i) the induction of TMP1 in yeast cells released from stationary phase did not require new protein synthesis, and ii) TMP1 mRNA levels increased in a gene dosage dependent fashion.

TMP1 expression in cells released from stationary phase, did not require de novo protein synthesis.

The accumulation of TMP1 mRNA, when stationary phase cells were stimulated to grow, was not prevented by the addition of cycloheximide. However, the concentration of cycloheximide used prevented incorporation of ^{35}S-methionine into acid precipitable materials (Figure 6B). This suggested that the accumulation of TMP1 mRNA, when stationary phase cells escaped G0 afrest, did not require de novo protein synthesis.

These results were therefore not consistent with a regulatory mechanism solely dependent on the periodic synthesis of a labile regulatory protein. Evidence suggesting the synthesis of an S phase specific regulatory "factor" has been observed in higher eukaryotes. The induction of thymidine kinase and thymidylate synthase mRNA, when quiescent 3T3 mouse cells are stimulated to

grow, is sensitive to cycloheximide. The accumulation of a labile protein has been shown to be required for the transcription of these 2 genes in 3T3 cells. Similarly the expression of the human histone H4 is induced by a trans-acting labile regulatory "factor" found only in S phase nuclear extracts (Coppock et al.,1984).

Nevertheless, we may not completely rule out the possibility that the regulation of the TMP1 gene is dependent on the periodic synthesis of a labile protein sometime after the "START" event. It is still possible to postulate that the mechanism which turns on the TMP1 gene is achieved by the interaction of two regulatory proteins. One of them would be a labile "repressor" of transcription and the other would be a protein periodically synthesized near "START" to inactivate the "repressor". Such a regulatory mechanism would still allow the transcription of TMP1 to resume in the presence of cycloheximide.

At least three different mechanisms could explain the accumulation of TMP1 mRNA in the presence of cycloheximide. These mechanisms include:

1) A posttranslational modification of a regulatory protein by a mechanism such as glycosylation, phosphorylation or dephosphorylation could regulate TMP1 expression. This posttranslational modification would inactivate or activate a regulatory protein which in turn

would regulate TMP1 transcription. Recent studies on the mechanism which controls the expression of the DHFR gene in mouse cells (Wu et al.,1982), have shown that transcription of DHFR was inhibited by high concentrations of cAMP. These findings led the authors to propose a mechanism regulating DHFR gene expression that would illustrate this first hypothesis. They proposed that the expression of DHFR gene could be regulated by the cell cycle stage dependent phosphorylation and dephosphorylation of a non-histone chromosomal protein by cAMP dependent protein kinase and phosphatase activities.

2) Cell cycle stage dependent changes in the stability of the TMP1 mRNA. This mechanism would imply that the periodic increase in the levels of TMP1 message during the cell cycle would be the result of a constant rate of transcription with differential stability of the message. Previous results obtained in our lab are not consistent with a cell cycle stage dependent stability of the TMP1 message. The fusion of a sequence 5' to the TMP1 gene with the structural portion of the E.coli lacZ gene was sufficient to confer a cell cycle dependent accumulation of beta-galactosidase activities (R.Ord,1987). In addition, a deletion mutation which maps 5' to the TMP1 transcriptional initiation sites abolished the cell cycle dependent expression of the TMP1'-'lacZ gene (R.Ord,1987). Furthermore, the addition

of cycloheximide results in the superinduction of genes that are regulated at the level of mRNA stability (i.e. human histone H4 , Sive et al.,1984). The results presented in Figure 6A, did not show superinduction of TMP1 mRNA levels in presence of cycloheximide [addition at t=(0)]. These results did not favor a cell cycle stage dependent stabilization of the TMP1 message.

3) Cyclohexamide inhibits the synthesis of a labile repressor protein whose presence is required to prevent the transcription of TMP1 .

The results obtained in the investigations of the regulatory mechanism(s) governing the TMP1 gene expression, could not distinguish between the first and the third hypothesis.

TMP1 mRNA levels and gene dosage were proportional.

The results presented in this study revealed that the levels of TMP1 mRNA increased as the number of copies of the TMP1 gene per cell were increased from 1 to 3 to almost 40. The increased levels of TMP1 mRNA were proportional to the TMP1 gene copy number (see Figure 8). Therefore the insertion of extra copies of the TMP1 gene did not trigger any regulatory mechanism which compensated for the extra TMP1 gene through either transcriptional or post-transcriptional controls. Previous studies in yeast have shown that increasing the gene

dosage of 7 different ribosomal proteins genes resulted in the proportional increase in message levels. (Warner et al. 1985).

In contrast, studies have revealed that yeast does compensate for extra copies of the periodically regulated histone genes H2A and H2B through a posttranscriptional regulatory mechanism (Osley et al.1981). These results clearly showed that there were differences between the mechanisms governing the regulation of TMP1 , H2A and H2B gene expression, even though these genes are all periodically transcribed in yeast.

The accumulation of thymidylate synthase (T.S.), in the low copy number transformant was proportional to the increase in the TMP1 mRNA levels. However, the levels of TMP1 mRNA in AH22-pTL1' (high copy number) were 40 times higher than the levels detected in AH22 while the levels of T.S. activity detected in these two strains differed by only 10 fold (M.Greenwood,1986) (table 3). This observation suggested that the efficiency of translation of the TMP1 mRNA might be reduced when mRNA levels are high. Thus, TMP1 gene expression might be under some posttranscriptional regulatory mechanism. There are at least 3 possible mechanisms by which the cell could achieve posttranslational regulation of the TMP1 gene. The three possible mechanisms are:

i) Differential distribution of the TMP1 mRNA within the cell. For example some transcripts might be sequestered within the nucleus. This could be achieved by differential polyadenylation or through the dilution of a factor necessary for the transport of TMP1 mRNA out of the nucleus. ii) Excess levels of thymidylic acid could modulate translation of TMP1 mRNA. (Autogenous regulation). iii) Excess TMP1 mRNA is efficiently translated, but T.S. activity is regulated by a posttranslational modification. The mechanism would be unable to efficiently activate large excess of T.S.

Additional data is required to distinguish between these three regulatory mechanisms.

SECTION4: Characterization of the TMP1'-'lacZ fusion junctions.

The fusion of various lengths of the *TMP1* 5' region to the *E. coli* *lacZ* structural gene showed that increasing levels of beta-galactosidase expression occured as the length of the structural portion of the *TMP1* gene increased (R.Ord,1987; M.Greenwood,1986). To ascertain that these result were not an artifact of the plasmid constructions, I sequenced three of these hybrid *TMP1'-'lacZ* fusion genes. The resulting sequence analysis confirmed the sequences expected for two of the three fusions (Figure 10, 11, and 12,). The third *TMP1'-'lacZ* fusion (pRS264i) differed from the expected sequence because it was missing 21 base pairs from the 3' terminus of the *TMP1* portion. However, the sequencing analysis did confirm that in pRS264i the correct reading frame was maintained as you proceed from the *TMP1* ATG codon used to initiate translation through into the *lacZ* sequences.

The results presented in this section confirmed that these three fusion genes had the *TMP1* promoter region fused to *lacZ* .

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