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Localization and Characterization of the Regulatory Regions Involved in TMP1 Expression

© Lydia Lee

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Sciences at
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ABSTRACT

Localization and Characterization of the Regulatory Regions Involved In TMP1 Expression

Lydia Lee

The yeast TMP1 gene encodes the enzyme thymidylate synthase, which catalyzes the synthesis of the DNA precursor thymidylate monophosphate (dTMP). In this study, the regulatory regions involved in TMP1 expression were characterized and localized by using hybrid gene fusions that contain the lacZ structural gene fused in frame to varying portions of the TMP1 gene. Three sets of gene fusions were analyzed. One set contained increasing amounts of the upstream region of the TMP1 gene, while the second set consisted of increasing amounts of the N-terminal coding information. The last set consisted of hybrid promoter fusions constructed from the iso-1-cytochrome c gene, CYC1, and TMP1. Analysis of β-galactosidase activity produced by these gene fusions revealed the following: (i) At least two cis-acting sequences required for gene expression are found within 270 bp region upstream of the TMP1 open reading frame. (ii) The TMP1 gene appears to have a weak promoter. (iii) A region within the TMP1 coding region is important for normal levels of gene expression. (iv) There appear to be two thymidylate synthase inactivation mechanisms that are batch culture stage specific.

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INTRODUCTION

Very little is known about how the cell cycle is regulated. One possible approach towards understanding the regulatory mechanisms controlling cell cycle division is to identify those proteins that are cell-cycle-dependent or periodically expressed. That is, the levels of these proteins accumulate during certain, but not other, stage(s) of the cell cycle. Our lab has previously shown that thymidylate synthase is one of these periodically expressed proteins. It was, therefore, the objective of my thesis research to study the regulation of the yeast gene encoding thymidylate synthase (TMP1).

The introduction of this thesis discusses the following three areas that are directly related to my thesis: (i) Thymidylate metabolism. (ii) Yeast promoter elements. (iii) Experimental approaches used to identify promoter elements.

I. Thymidylate Metabolism

A model system to study the periodic regulation of gene expression and the regulatory mechanisms controlling cell cycle division is the yeast, Saccharomyces cerevisiae. In S. cerevisiae, the TMP1 gene encodes thymidylate synthase (Taylor et al, 1982), an enzyme that is essential for the synthesis of the DNA precursor thymidylate (dTMP), by catalyzing the reductive methylation of deoxyuridylate (dUMP). This reaction provides S. cerevisiae with its only source of thymidylate since wild type yeast is impermeable to dTMP (Bisson and Thorner, 1982), and cannot use thymine

or thymidine since it lacks a kinase for phosphorylating thymidine (Grivell and Jackson, 1968). Further, the perturbation of intracellular pools of dTMP has been shown to cause thymidylateless death (TLD), thymidylate excess death (TED), recombinogenesis and mutagenesis (for a review, see Barclay et al, 1982). One reason why yeast might be very sensitive to treatments which elevate intracellular thymidylate pools is that yeast apparently does not have the phosphorylases necessary for the catabolism of thymine nucleotides (Hammer-Jaspersen, 1983). Taking the above into consideration it is probably critical that yeast carefully regulate the production of thymidylate.

Present evidence suggests that the intracellular levels of thymidylate are controlled mainly by precisely regulating the levels of thymidylate synthase. This regulation includes the fact that the level of thymidylate synthase is periodically regulated during the cell cycle. Also, the low level of thymidylate synthase activity detected is near rate limiting for DNA synthesis. Hence, it has been speculated that the synthesis of thymidylate synthase plays an important role in regulating thymidylate metabolism.

Previous work has shown that TMP1 expression is periodic during the mitotic cell cycle (Storms et al, 1984). Both TMP1 mRNA and thymidylate synthase levels increase during the late G1 stage just prior to S phase, peak during the S phase, and then decline before the next cell division cycle begins. Further work indicated that cell cycle dependent

regulation of thymidylate synthase levels occurs by both transcriptional (MacIntosh, pers. comm.) and post-translational (Greenwood et al, 1986) mechanisms.

II. Yeast Promoter Elements

The primary level of regulation for protein-encoding genes occurs mainly by controlling the frequency of transcription initiation for mRNA synthesis (Serfling et al, 1985; Guarente, 1984). In S. cerevisiae, the mechanism of transcription initiation is not well understood. Elucidation of this process had been hindered by the lack of an in vitro transcription system. To circumvent this problem, three approaches were used: (i) defining the cis-acting target sites or promoter region of the gene in interest, (ii) identifying and purifying the trans-acting factors which recognize these target sites, and (iii) analysis of the conformational changes that the chromatin structure undergoes to become transcriptionally competent.

These studies indicated that yeast promoters are generally complex and composed of multiple elements. These elements include upstream activating sequences (UAS), upstream repressor sites (URS) or operator sites, TATA boxes, and initiator (I) elements. These cis-acting elements specify the levels of expression, the sites for initiation of transcription, the induction of transcription, and the repression of transcription.

Positive control

The UAS elements are small regions, between 15 and 40 base pairs (bps) in length, located in the range of 80 to 1400 bps upstream of the mRNA transcription start sites. These elements have been found to be critical for transcription, and for regulating transcription. Also, UAS elements can confer transcriptional regulation on other genes (Struhl, 1986a). Essentially, UAS elements appear to be the cis-acting sites involved in positive control of gene regulation.

UAS elements appear to be protein binding sites which are recognized by specific DNA binding proteins. These proteins are trans-acting factors involved in activating gene expression. Presently, it is believed that these proteins are specific transcription factors that recognize the DNA target sites and promote transcription.

The yeast transcriptional activators, GCN4 which is required for the coordinate derepression of a set of amino acid biosynthetic genes during amino acid starvation, and GAL4 which is required for the induction of the galactose utilization genes (GAL1,10,7) have been shown to bind directly to specific DNA sequences within their cognate UAS regions (Bram and Kornberg, 1985; Giniger et al, 1985; Hope and Struhl, 1986). Also, the DNA binding and transcriptional activation properties of these two activators are separable as functional domains of the protein molecule (Hope and Struhl, 1987; Keegan et al,

1986). Comparison of the amino acid sequences in the transcriptional activation domains reveals no similarity except these domains are highly acidic. Because RNA polymerase II does not bind DNA *in vitro*, these domains have been proposed to interact with the β-subunit of RNA polymerase II thereby positioning it for transcription. Similarly, it is also possible that intermediate proteins interact with both RNA polymerase II and the UAS binding protein.

A comparison of the DNA sequences encoding UAS activity reveals no similarity unless the genes are co-regulated. Generally, in the upstream regions of co-regulated genes, a consensus sequence is found which is the target site for sequence-specific DNA binding proteins. For instance, GCN4 protein binds the sequence 5'-TGACTC-3'. This sequence is found in the upstream region of amino acid biosynthetic genes which are under the general control of nitrogen metabolism, while the GAL4 protein interacts with several 17 bp consensus sequences which are found in the upstream sequences adjacent the GAL1,10,7 genes.

However, similarity between UAS sequences may not be enough for specifying binding by the DNA sequence specific binding proteins. MATα1, an activator of α-specific genes (α-sgs), acts synergistically with another *trans*-acting factor, PRTF (P box recognition transcription factor which is also known as pheromone/receptor transcription factor). Apparently, PRTF may be the actual transcription activator

for cell type specific genes since this factor is found in all cell types a, α, and a/α. The P box which is the binding site for PRTF is a perfect 16 bp palindromic sequence upstream of a-specific genes (a-sgs) but is an imperfect palindrome in α-sgs. Consequently, it has been proposed that the MATα1 protein is required as an aid for PRTF to recognize the divergent P box for α-sg expression since no known additional factor is required for a-sgs expression (Bender and Sprague, 1987; Jarvis et al, 1988).

In striking contrast to the UAS binding proteins described above, the HAP1 transcriptional activator apparently can bind to two different DNA sequences found in the upstream regions of the iso-cytochrome c genes, CYC1 and CYC7 (Pfeifer et al, 1987). However, the nature of the protein-DNA recognition between HAP1 and these upstream sequences is unknown.

The yeast UAS is analogous to the mammalian enhancer in that it can function bidirectionally, and can operate at a variety of distances when placed upstream of the transcription initiation sites of a gene (Serfling et al, 1985). In contrast to mammalian enhancer sequence, the yeast UAS elements do not function downstream of transcription initiation (Guarente and Hoar, 1984). Nonetheless, recent results suggest that the basic mechanism of transcriptional activation is the same in all eukaryotes (Guarente, 1988). For example, the transcriptional activation domain of the yeast GAL4 protein can promote gene

expression in both the chinese hamster ovary and HeLa cell lines (Kakidani and Ptashne, 1988; Webster et al, 1988). Activation of transcription by the intact GAL4 protein was dependent on the presence of the GAL4 UAS, or else required the construction of a hybrid protein consisting of the GAL4 DNA binding domain and the transcriptional activation domain of the estrogen receptor. Conversely, hybrid protein fusions containing the mammalian fos protein fused to lexA protein activate gene expression in yeast if the lexA binding sequence is appropriately positioned upstream of the transcription start signals (Lech et al, 1988).

These studies indicate that UAS elements are the DNA target sites for positive control of gene expression. The site-specific DNA binding proteins confer promoter specificity and appear to be the factors that respond to physiological conditions to effect gene expression.

Models for transcriptional activation

Four models for the mechanism by which transcriptional activation complexes function have been proposed: looping, sliding, oozing, and twisting (Ptashne, 1986). In general, these models are centralized around the theme that transcription initiation commences by the specific interaction of DNA binding proteins to the UAS region of the promoter. These models attempt to explain how regulatory proteins at the UAS activate transcription.

In the DNA looping model, the intervening DNA between the UAS and the transcription initiation region is looped

out by the interaction of the DNA binding proteins bound at their cognate DNA sites which can be situated far apart in the promoter region. This pre-initiation complex then interacts with other trans-acting factors (e.g. RNA polymerase II, initiation factors) in a protein-protein interaction to form the transcription initiation complex which initiates mRNA synthesis. In the sliding model, the DNA transcription factors initially interact at the UAS, and then traverses from the UAS to another site where another factor, possibly RNA polymerase II, becomes associated for transcriptional activation. Oozing involves a cascading succession of protein interactions that are initiated by a specific protein binding at the UAS and then additional proteins bind in an hierarchical fashion as the complex moves towards the site for transcription initiation. Lastly, twisting suggests that regulatory proteins recognize altered forms of DNA, such as left-handed or single stranded DNA. For this last model, enzymatic modification of the chromatin by the regulatory proteins has also been postulated. Although sliding, oozing, and twisting models have not been completely discounted, there is a growing body of evidence that suggests that transcription initiation generally occurs by DNA looping (Ptashne, 1985).

Negative control

A few examples of negative regulation are known in yeast. The best characterized example is $\alpha 2$ dependent repression of a-sgs. Other less characterized examples

include the cell cycle dependent genes HO and H2B-H2A, the silent mating type loci HMLα and HMRa, and the catabolite repressible genes GAL1,10,7. These studies indicated that the negative cis-acting elements can be found upstream, downstream, or overlapping a gene's UAS element. These negative cis-acting elements are putative sites for DNA binding proteins. This has been shown to be the case for one regulatory protein, the MATα2 repressor (Johnson and Herskovitz, 1985). Although negative regulation is not well characterized in yeast, these studies suggest there may be different mechanisms of repression.

Most studies, at present, have identified the DNA segment within which the negative site resides in the promoter region. A few studies have also identified the negative factor(s) by genetic or biochemical analysis. These studies indicate that the negative control sites can reside elsewhere far from the UAS and still confer its repressing effect (Johnson and Herskowitz, 1985; Struhl, 1985; Brand et al, 1985). This suggests that the prokaryotic form of repression, which involves steric hindrance or competition, is not the only repression mechanism acting in yeast. At present, repression of the a-specific gene BAR1 by the α2 protein is the only example of direct steric hindrance (Kronstad et al, 1987). Both the negative site (α2 operator) and UAS overlap, indicating that the α2 repressor prevents transcription initiation by occluding the binding by some positive transcription factor

(possibly the PRTF factor mentioned, see above).

In contrast, the α2 operator in the STE6 gene is located between the UAS and TATA elements. From hybrid promoter constructions of the α2 operator and the CYC1 promoter, Johnson and Herskowitz (1985) showed that when the α2 operator is located upstream of the CYC1 upstream activating sequences (UAS_C) CYC1-lacZ expression was repressed, although repression was 10-fold more effective when operator was located between the UAS_C and TATA site. This negative element has characteristics similar to UASs in that it can function bidirectionally, and can exert its effect when placed upstream of the UAS. This result indicates that negative regulation in yeast is not necessarily mediated by a steric hindrance. Consequently, Struhl (1986a) has proposed that positive and negative control in yeast may occur by similar mechanisms which have opposite effects on expression. However, the nature of this repression is not well understood.

Another possible form of negative regulation is the blockage effect, whereby the repressor binds at a site between the UAS and RNA start site and occludes the transcription apparatus from traversing to the site of transcription initiation. This has been shown artificially by Brent and Ptashne (1984) using the bacterial repressor, the lexA protein and its DNA binding site, the lexA operator, as a model system to elucidate the process of repression. This study showed that the location of the lexA

operator between the GAL1 upstream activating sequence (UAS_G) and TATA sites did not by itself obstruct GAL1 transcription but repressed expression if the lexA operator was bound by the lexA protein. However, repression did not occur when the lexA operator was placed upstream of the UAS_G irrespective of the presence or the absence of the lexA repressor. This suggests that initiation of GAL1 transcription occurs by a sliding mechanism (Brent, 1985); however, this is an artificial system that uses a bacterial repressor that may differ from the eukaryotic mechanisms of repression.

A novel mechanism of repression is observed for the silent mating type loci HMLα and HMRa (or HM loci). Repression of these loci appears to require progression through DNA synthesis or S phase of the cell cycle (Miller, 1984). The cis-acting negative HMLE and HMRE (E sites), located 1 kb from their respective HM loci, and the action of four trans-acting SIR gene products are essential for negative regulation. The E site behaves as a negative enhancer, in that it functions bidirectionally, is position-independent, and represses heterologous genes at distances as great as 2.5 kb (Brand et al, 1985). Because this region has a functional autonomously replicating sequence (ARS), it has been speculated that DNA replication is essential for transcriptional repression of the HM loci (Brand et al, 1987). This kind of enhancer-like regulation may have evolved to inactivate genes that have all the promoter

information necessary for transcriptional competence.

Neither HO nor the galactose catabolism genes GAL1,7 and 10 appear to contain distinct negative control sites (Nasmyth, 1985; Guarente, 1984). Their negative regulation appears to be mediated by the direct antagonism of the positive transcription factor by the repressor. Expression analysis of the HO gene has indicated that its negative regulation is mediated by the direct antagonism of the positive trans-acting SWI factors by the negative SIN factors (Sternberg et al, 1987). Similarly, in vivo and in vitro studies of the negative regulator for the galactose metabolism genes (the GAL80 protein), indicates that its site of action appears to be the transcriptional activation domain of the GAL4 protein (Lue et al, 1987; Selleck and Majors, 1987). In the absence of the GAL4 product, GAL80 cannot be detected at the promoter region of the GAL1 and GAL10 genes. This strongly suggests that the negative effect is mediated by a protein-protein interaction.

The TATA element

In yeast, the TATA (canonical sequence TATAAA) element does not appear to be a critical promoter element as it is in higher organisms and in E. coli. In higher organisms, the TATA element is invariably located at 25 to 30 bp from transcription start site, and is required for specifying the correct RNA start sites. TATA sequences in yeast promoter regions can be located at variable distances in the range between 40 to 120 bp upstream from the mRNA initiation start

sites. In addition, there is not an absolute requirement for TATA elements since the highly expressed PGK (phosphoglycerate kinase) gene does not contain a TATA promoter element (Ogden et al, 1986).

At least two distinct classes of TATA elements have been identified within the HIS3 promoter by Struhl (1986b). One element is used for basal and the other is used for induced expression. This study suggested that the TATAAA sequence may be an integral component of a regulated promoter while the divergent versions correspond to constitutive expression. However, the inverse relationship seems to occur in higher organisms. The human hsp70 TATAA element is responsive to E1A inducibility and can be replaced by TATAA sequences supplied by other genes but will not respond if substituted by a divergent version such as the SV40 TATTTAT sequence (Celeste Simon et al, 1988). Also, recent experiments suggest that this site interacts with a DNA binding protein, the TATA binding factor.

Together, these findings suggest the TATA element may be the target site for factors which determine promoter strength. These factors may be different classes of general transcription factors (analogous to the different sigma factors in E. coli) which govern the rates of RNA synthesis.

Initiator elements

Lastly, the initiator elements are found near the transcript start sites. Although this element does not affect the rate of RNA synthesis, it is the primary

determinant of the mRNA start sites. Because this element is very close to the transcript start sites, it may be the target site where RNA polymerase II binds. However, there is no biochemical evidence to support this hypothesis. The prevailing view is that RNA polymerase II does not interact directly with DNA during transcription initiation but must be complexed with a trans-acting factor that does (Burton et al, 1986).

Overall, transcription initiation in yeast appears to be a complex interaction of trans-acting factors which interact with DNA at multiple target sites to regulate gene expression. UAS elements are the cis-acting sites for positive regulation of gene expression. They are the recognition sites for binding by specific DNA binding proteins. Both the UASs and URSs appear to be the major determinants for regulating gene expression. The other promoter elements, the TATA box and the I element, are also potential sites for regulating gene expression but appear to be target sites for general transcription factors involved in transcriptional activation. The development of the in vitro yeast transcription system would help to elucidate the components involved in the initiation of transcription for mRNA synthesis (Lue and Kornberg, 1987).

III, Experimental approaches used to identify promoter elements

Localizing the cis-Acting Sequences

The cis-acting sites were generally identified by deleting progressively the genetic information in the 5' intergenic region and assessing the effect of these deletions on gene expression. The effect of the deletion can be quantitated directly by measuring RNA levels, or indirectly by measuring the protein levels with an enzymological assay. Alternatively, the effect on gene expression can be measured indirectly by following the expression levels of another gene product.

Many researches have used the lacZ gene for studies of this sort (Casadaban, 1983). These gene fusions used the E. coli lacZ gene which lacks its promoter, ribosome-binding site, and ATG initiation codon. Provision of these transcriptional and translational control signals by sequences from another gene results in the synthesis of β-galactosidase which can be easily assayed colorimetrically. Since the expression of β-galactosidase from these hybrid genes is dependent on the regulatory signals of the other gene, deletion analysis of the DNA sequences can be used to identify the cis-acting regulatory sites.

This approach has been used to determine the regulation of genes involved in biological processes such as response to amino acid starvation, determining mating or cell type control, response to changing carbon sources, and cell

division.

Experimental Strategy

In this thesis, a study of the yeast TMP1 regulatory regions was determined by using hybrid gene fusions that contained varying portions of the TMP1 gene spliced to the coding region of the 'lacZ gene. Different portions of the TMP1 5' intergenic and N-terminal coding regions were used to express 'lacZ. This approach enabled me to delimit the regulatory regions involved in controlling TMP1 expression.

In this thesis the following questions were addressed.

1. Is all the information for normal levels of TMP1 gene expression contained within the 376 bp of upstream information flanking the open reading frame? To answer this question, more information was retrieved from yeast DNA and subcloned into two types of TMP1'-'lacZ gene fusions. One type contained 15 and the other 112 codons of thymidylate synthase N-terminal coding information. The expression of the parental and the derived plasmids were compared by determining the levels of fusion protein expressed.
2. Can the cis-acting sequences defined above confer their regulatory properties to another gene, a CYC1'-'lacZ fusion gene? Hybrid promoter plasmids lacking functional UAS activity and ones containing the putative TMP1 UAS were constructed. The levels of β-galactosidase expression were compared with the parental CYC1'-'lacZ gene fusion as well as two other TMP1'-'lacZ gene

fusions.

3. Does the level of β-galactosidase expressed from the TMP1'-'lacZ gene fusions reflect the levels of thymidylate synthase expressed from the TMP1 gene? Comparative analysis was standardized by calculating the number of peptide monomers produced by the gene fusions and the TMP1 gene.
4. β-galactosidase levels from the hybrid gene fusions containing varying portions of the 5' intergenic region and from the hybrid promoter gene fusions suggested that the TMP1 gene has a very weak promoter because these gene fusions did not express lacZ at levels near the level at which the TMP1 gene is expressed. To determine whether information in the TMP1 coding region specified enhanced levels of expression, I also analyzed the expression from the hybrid gene fusions carrying varying portions of the TMP1 coding region.
5. Within the TMP1 open reading frame, a regulatory region has been identified that responds to post-translational inactivation during the later stages of batch growth. To confirm that this post-translational regulatory mechanism is a general feature of its regulation, the levels of β-galactosidase were determined from the transformants at low density batch cultures. That is, I wanted to test whether this post-translational mechanism was operational throughout all stages of batch culture growth.

MATERIALS AND METHODS

Plasmid constructions

New plasmids were constructed by digesting parent plasmids with the appropriate restriction endonucleases, mixing in the molecular proportions of 2 insert to 1 vector plasmid, reannealing, and ligating with T4 DNA ligase (Boehringer). See table 1 for a list of the plasmids constructed and donated for use in this study.

Strains

The E. coli strains JF1754 and JM83 (see table 2) were used as host strains for selecting newly constructed plasmids or for propagating plasmids. JF1754 was used as host strain for plasmids that contained the yeast LEU2 gene because LEU2 complements the defective leuB gene. JM83 was used for the recombinant plasmids derived from the pUC18 or pUC19 plasmid. S. cerevisiae strain AH22 was used as the host strain for autonomously replicating plasmids and integrating plasmids. Transformants containing integrating plasmids are listed in table 2. Yeast strains containing autonomously replicating plasmids have not been listed. These transformants will be designated in the text by a T followed by plasmid name.

Growth media

E. coli strains were grown in either minimal M9 (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, 17 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, 20 μg/ml thiamine-HCl, and 50 μg/ml of the required amino acids), or rich LB (1% N-Z amine

TABLE 1: List of plasmids used

Plasmid	Description	Source
pUC18	amp^R ori; lacZ (2.686 kb)	J. Messing
pYF91	amp^R ori; 2μm LEU2 (13.4 kb)	R. K. Storms
pTL1	amp^R ori; 2μm LEU2 TMP1 (23.4 kb)	G. R. Taylor
pTL221	amp^R ori; TMP1 (7.3 kb)	G. R. Taylor
pRS464	amp^R ori; TMP1 URA3 (8.45 kb)	P. P. Poon
pRS479	amp^R ori; TMP1 URA3 (9.2 kb)	this study
pRS820	amp^R ori; TMP1 (3.6 kb)	this study
pRS829	amp^R ori; TMP1 (5.0 kb)	this study
pRS2043	amp^R ori; TMP1 (2.861 kb)	C. Brideau
	Autonomous replicating gene fusion plasmids	
pYT760-ryp3	amp^R ori; 2μm LEU2 ryp3-lacZ (15.64 kb)	D. Thomas
pLG669-Z	amp^R ori; 2μm URA3 CYC1-lacZ (11.8 kb)	L. G. Guarente
pRS845	amp^R ori; 2μm LEU2 TMP1-lacZ (18.8 kb)	R. K. Storms
pRS535	amp^R ori; 2μm LEU2 TMP1-lacZ (17.45 kb)	R. W. Ord
pRS954	amp^R ori; 2μm LEU2 TMP1-lacZ (17.5 kb)	Y. Ho
pRS872	amp^R ori; 2μm LEU2 TMP1-lacZ (17.6 kb)	Y. Ho
pRS269	amp^R ori; 2μm LEU2 TMP1-lacZ (17.65 kb)	R. W. Ord

List of plasmids used (cont'd)

Plasmids	Description	Source
pRS759	amp^R ori; 2μm LEU2 TMP1-lacZ (17.9 kb)	M.T. Greenwood
pRS840	amp^R ori; 2μm LEU2 TMP1-lacZ (19.05 kb)	this study
pRS1034	amp^R ori; 2μm LEU2 TMP1-lacZ (18.25 kb)	this study
pRS822	amp^R ori; 2μm LEU2 TMP1-lacZ (18.45 kb)	this study
pTL30	amp^R ori; 2μm LEU2 TMP1-lacZ (17.3 kb)	G. R. Taylor
pTL31	amp^R ori; 2μm LEU2 TMP1-lacZ (17.1 kb)	G. R. Taylor
pTL32	amp^R ori; 2μm LEU2 TMP1-lacZ (17.2 kb)	G. R. Taylor
pTL35	amp^R ori; 2μm LEU2 TMP1-lacZ (17.2 kb)	G. R. Taylor
pRS741	amp^R ori; 2μm LEU2 TMP1-lacZ (17.4 kb)	R. W. Ord
pRS744	amp^R ori; 2μm LEU2 TMP1-lacZ (17.4 kb)	R. W. Ord
pRS669	amp^R ori; 2μm LEU2 TMP1-lacZ (17.5 kb)	R. W. Ord

List of plasmids used (cont'd)

Plasmid	Description	Source
	Integrating gene fusion plasmids	
pRS306	ori; LEU2 TMP1-lacZ (12.9 kb)	this study
pRS448	amp^R ori; LEU2 TMP1-lacZ (15.4 kb)	this study
pRS463	amp^R ori; LEU2 TMP1-lacZ (13.8 kb)	this study
	Autonomously replicating hybrid promoter fusion plasmids	
pRS2101	amp^R ori; 2μm LEU2 CYC1(▲UASc)-lacZ (18.35 kb)	this study
pRS2102	amp^R ori; 2μm LEU2 TMP1-CYC1-lacZ (18.5 kb)	this study
pRS2103	amp^R ori; 2μm LEU2 TMP1-CYC1-lacZ (18.5 kb)	this study

TABLE 2

List of strains used

A. E. coli

Strain	Description	Source
JF1754	hsdr lac gal metB leuB hisB436	J. Friesen
JM83	ara Δlac pro thi strA φ80dlacZM15	J. Messing

B. S. cerevisiae

Strain	Description	Source
AH22	MATa can1-1 leu2-3,-112 his4-519	G. R. Fink
M12B	MATα trp1-289 ura3-Δ52	T. G. Cooper
RS456	AH22 [pRS306 (2 copies) :: TMP1[a]	this study
RS457	AH22 [pRS306 (2 copies) :: TMP1[a]	this study
RS458	AH22 [pRS306 (1 copy) :: leu2[a]	this study
RS561	AH22 [pRS463 (1 copy) :: TMP1[a]	this study
RS566	AH22 [pRS463 (5 copies) :: leu2[a]	this study
RS567	AH22 [pRS463 (4 copies) :: leu2[a]	this study

a. notation adopted to indicate the number and the plasmid sequence integrated at the chromosomal locus

type A, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose, pH 7) media. Generally, strains were grown in minimal media lacking the appropriate amino acid when selecting a recombinant plasmid, or were grown in rich media LB with ampicillin (20 μg/ml) when isolating plasmid DNA. Strain JF1754 transformed with plasmids bearing the yeast LEU2 gene was grown in minimal media supplemented with the amino acids methionine and histidine. JM83 transformed with pUC18 or pUC19 recombinant plasmids was grown on minimal media supplemented with 50 μg/ml of proline, 20 μg/ml of ampicillin, and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal).

Yeast strains were grown in minimal YNBD (0.67% Difco Yeast Nitrogen Base w/o amino acids, 2% glucose, 50 μg/ml of required amino acids) or rich YEPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) media. Strains transformed by autonomously replicating plasmids were selected and maintained by growth in minimal media lacking leucine (YNBDH). Strains transformed by integrating plasmids were selected by growth in YNBDH. Once obtained, subsequent growth was in either YNBDH or YEPD.

Transformation

Routinely, 100 ml cultures of E. coli were grown to A_{600} 0.2 (LKB Ultraspec) for transformation. The flask was cooled immediately by immersion in a chilled ice - water bath. Cells were harvested, washed once with 50 mM $CaCl_2$, and then resuspended in 1/20th volume of the original

culture with 50 mM $CaCl_2$/10% glycerol. The amount of competent cells required for the current transformation was removed, and the rest dispensed into 1 ml or 0.2 ml aliquot for storage at -80°C.

Yeast AH22 cells were transformed by either the LiCl protocol (Ito et al, 1983) when using autonomously replicating plasmids, or the spheroplast technique (Hinnen, 1978) when using integrating plasmids. Spheroplasting yeast cells for integration of plasmid sequences was preferred since the transformation frequency was much higher. Typically, 1 μg of autonomously replicating plasmid DNA, and 10 μg of integrating plasmid DNA was used to transform yeast cells. To enhance the frequency of plasmid integration, DNA was linearized by a restriction endonuclease that cleaved a site within the TMP1 or LEU2 sequences for targeting to either of the two chromosomal sites (Orr-Weaver et al, 1981).

DNA Isolation

Small scale and large scale plasmid DNA preparations were isolated from E. coli using the protocol developed by Birnboin and Doly (1979). Large scale plasmid preparations were purified by CsCl-ethidium bromide centrifugation.

Plasmid DNA was isolated from yeast using the same procedure as for E. coli except that yeast cell walls were digested with Zymolyase (see below). Total chromosomal DNA was isolated and purified from yeast using the procedure by Davis et al (1980) with the following modification. 25 to

100 ml of yeast culture was grown to stationary phase, harvested, and washed once with 1 M sorbitol or lytic solution (1 M Sorbitol, 20 mM KH_2PO_4 pH 6.8, 50 mM EDTA). Cells were spheroplasted in 1/10th volume of lytic solution with 10 µg/ml of Zymolyase 100T (Miles), and 0.1% of β-mercaptoethanol. The conversion of cells into spheroplasts was determined by the addition of water or 0.1% SDS, and observing microscopically for the loss of intact cells. Spheroplasts were washed once with 1 M sorbitol and the pellet resuspended in 50 µg/ml of Proteinase K and 0.5% SDS (Maniatis, 1982). After overnight digestion at 37°C, 1/10th volume of chilled 5 M K acetate pH 6.0 was added and left on ice for one hour before spinning down at an average relative centrifugal force (RCF) of 17000 X g (IEC). The supernatant was transferred to another 50 ml Oakridge tube and an equal volume of 10 M LiCl was added. This was chilled for one hour to precipitate the bulk of RNA and proteins before centrifuging at an average RCF of 17000 X g (IEC). The supernatant containing the DNA was transferred again into a fresh Oakridge tube for ethanol (EtOH) precipitation. Chromosomal DNA was essentially free of RNA and proteins but RNAse treatment and phenol extraction removed all traces.

DNA hybridization

Two methods were used for DNA hybridization: Cooper's method (1982) or desiccated gel protocol of Tsao and Pearlman (1983). When using Cooper's method, Pall membranes were used instead of nitrocellulose. Otherwise, DNA

hybridization was performed as described.

DNA hybridization by the dried gel method is a modification of the procedure developed by Tsao and Pearlman (1983). In general, chromosomal DNA was denatured in a 0.5 M NaOH - 1.5 M NaCl solution (three changes for 15 minute incubations) with gentle shaking to completely immerse the gel. The gel was neutralized in 0.5 M Tris pH 7.5 - 1.5 M NaCl (two changes for 30 minutes incubations). The gel was immediately placed in a BioRad slab gel drier and was vacuum dried to paper thinness overnight at 80°C.

When ready to hybridize, the dried gel was rehydrated in filtered water for 5 minutes. The gel was then placed into a heat-sealable bag (Seal-A-Meal™) with prehybridization solution (5X SSPE [0.9 M NaCl-0.05 M NaH_2PO_4-5 mM EDTA pH 7.0] - 5X Denhardt's [0.5% Ficoll-0.5% polyvinylpyrrolidone-0.5% BSA] - 0.1% SDS) for at least 2 hours at 65°C. After prehybridization, heat-denatured radioactively labelled DNA was added and incubation continued overnight. After hybridization, the gel was washed twice with 2X SSPE - 0.1% SDS for one to two hours, and once with 1X SSPE for 2 hours at 65°C. The hybridization bag was then placed into a 45°C bath for 15 minutes and finally at room temperature for 5 minutes. The gel was placed in a DuPont Lightning Plus intensifier screen to expose X-ray film (Kodak X-OMAT AR). This was stored at -80°C for 1 to 7 days depending on the specific activity of the radioactively labeled DNA fragment (see below).

DNA fragment isolation for nick translation

Both the 954 bp HindIII to BamHI TMP1 fragment and the 2.2 Kb SalI to XhoI LEU2 fragment were purified from agarose gels by electroeluting the bands onto dialysis membrane, essentially as described by Maniatis (1982) with the following modifications. 10 µg of plasmid DNA (either pRS479 for TMP1 or pYF91 for LEU2) was digested by restriction endonucleases and loaded into the 3 cm slot of an agarose gel. Electrophoresis was carried out until the bands were separated. The DNA bands were stained lightly by 0.1 µg/ml ethidium bromide in TAE buffer (0.04M Tris-acetate - 0.002M EDTA, pH 8.3). An incision was made in front of the desired band to insert a dialysis membrane and 3 MM Whatman filter paper. The 3 MM Whatman paper was placed between the DNA band and dialysis membrane. This gel was returned to the electrophoresis tank to transfer DNA from the gel to the dialysis membrane. Both the dialysis membrane and Whatman filter paper were removed and inserted into a perforated 400 µl microfuge tube fitted inside a 1.5 ml microfuge tube. The eluate was recovered by 15 to 30 second centrifugation at 12000 X g (Ependorf). The membrane was washed twice with 100 µl of TAE, and the eluate and washes were pooled. The NaCl concentration was adjusted to 0.2 M and the DNA was purified by passage through an Elutip-d^{TM} column (Schleicher & Schuell). After EtOH precipitation, the DNA was resuspended in 20 to 25 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA).

0.5 to 1 μg of DNA fragment was labeled to a specific activity of 10^6 - 10^7 cpm/μg using the BRL nick translation kit with either [α-^{32}P]dATP (ICN), [α-^{32}P]dGTP (Amersham), or [α-^{32}P]dCTP (NEM). If P^{32} incorporation was greater than 30%, T4 DNA ligase was added to repair nicks in the DNA fragment as recommended by Maniatis.

β-galactosidase determination

Routinely, either 9 ml aliquot (from strains with autonomously replicating plasmids) or 25 ml aliquot (from strains with integrated plasmids) were harvested from batch cultures and used for β-galactosidase assays. Yeast cultures were usually grown for 20 to 23 hours in YNBDH media (approximately 8 to 10 generations) before samples were harvested for β-galactosidase determination. Duplicate samples were taken from each culture. After centrifugation, pellets were frozen in liquid nitrogen before storing at -80°C. Cell numbers were determined spectroscopically (A_{600}).

β-galactosidase activity from yeast was determined essentially as described by Miller (1972), except that 3% Brij-35 was included in the Z-buffer to permeablize cells. Experiments were repeated, at least once, to evaluate the relative level of β-galactosidase activity expressed by the different transformants.

RESULTS

I. RETRIEVAL OF ADDITIONAL TMP1 UPSTREAM SEQUENCES

The yeast TMP1 gene has been cloned on a 9.6 kb HindIII fragment (Taylor et al, 1982). Complementation analysis revealed that the TMP1 gene was located near one end of this HindIII fragment. Also, DNA sequencing indicated that this fragment contained only 376 bp of information upstream of the open reading frame encoding thymidylate synthase. Since Nasmyth (1985) found that the HO gene, another cell cycle regulated gene, required at least 1400 bp of upstream information for its correct expression, I reasoned that the TMP1 gene may require more than 376 bp of upstream information for its correct expression. Therefore, before I began my studies into localizing and characterizing the sequences regulating TMP1 expression, I wanted to retrieve from yeast more of the DNA sequences from upstream of the TMP1 gene.

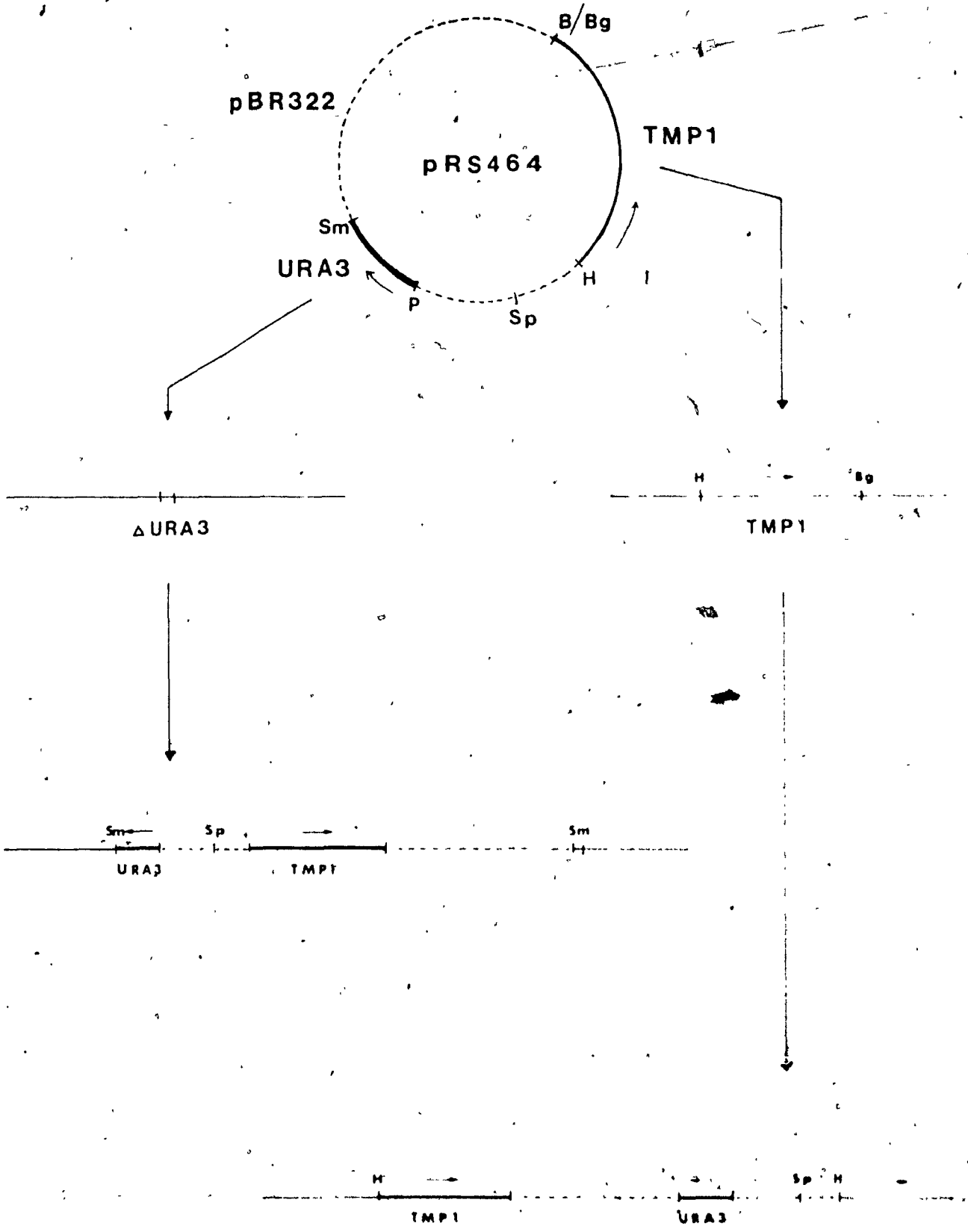
The strategy used to retrieve more DNA from upstream of the TMP1 gene is outlined in figure 1. The basic features of this strategy included: (i) Transformation of yeast M12B with an integrating plasmid (pRS464) carrying the TMP1 sequences to allow integration by homologous recombination at the TMP1 locus. (ii) Identification, using the DNA blot analysis, of the transformants with the integrating plasmid present at the TMP1 locus (data not shown). (iii) Retrieving the plasmid and additional sequences flanking the TMP1 gene from these yeast transformants.

FIGURE 1

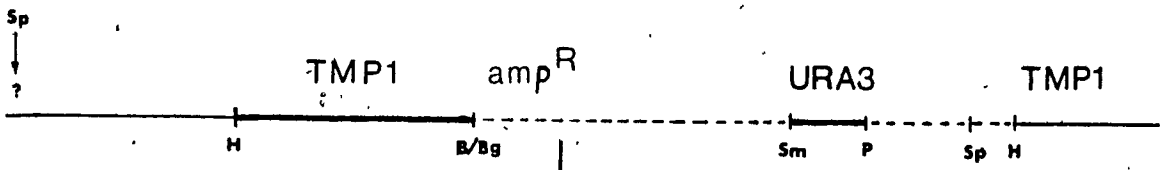
Strategy to retrieve more TMP1 upstream information

A: Primary steps: Plasmid pRS464 transformed into strain M12B integrates at either TMP1 or Δura3 locus to form URA3 transformants. Shown are the partial restriction maps of the plasmid pRS464, the chromosomal TMP1 and Δura3 regions, and the predicted maps of these regions after plasmid integration. The thin line (——) depicts chromosomal DNA; the thick line (▬) depicts plasmid TMP1 and URA3 sequences; and the dashed line (---) depicts pBR322. The sites for HindIII, SphI, PstI, SmaI, and BamHI/BglII are indicated by H, Sp, P, Sm, and B/Bg respectively. The arrows indicate direction of transcription.

B: Schematic diagram depicting the strategy used to retrieve additional information from upstream of the TMP1 gene. Shown at the top is a partial restriction map of a M12B transformant containing one copy of pRS464 integrated at the TMP1 locus. The thin line (——) depicts chromosomal DNA; the thick line (▬) depicts TMP1 and URA3 plasmid sequences; and the dashed line (---) depicts pBR322 DNA. The sites SphI, HindIII, BamHI/BglII, SmaI, and PstI are indicated by Sp, H, B/Bg, Sm, and P respectively.


B/Bg
pBR322
pRS464
TMP1
Sm
URA3
P
Sp
H
ΔURA3
H
Bg
TMP1
Sm
Sp
Sm
URA3
TMP1
H
Sp
H
TMP1
URA3

1B.



digestion by SphI into smaller DNA fragments

ligation with T4 DNA ligase

transform E. coli JF1754

select ampR clones

isolation of plasmid DNA

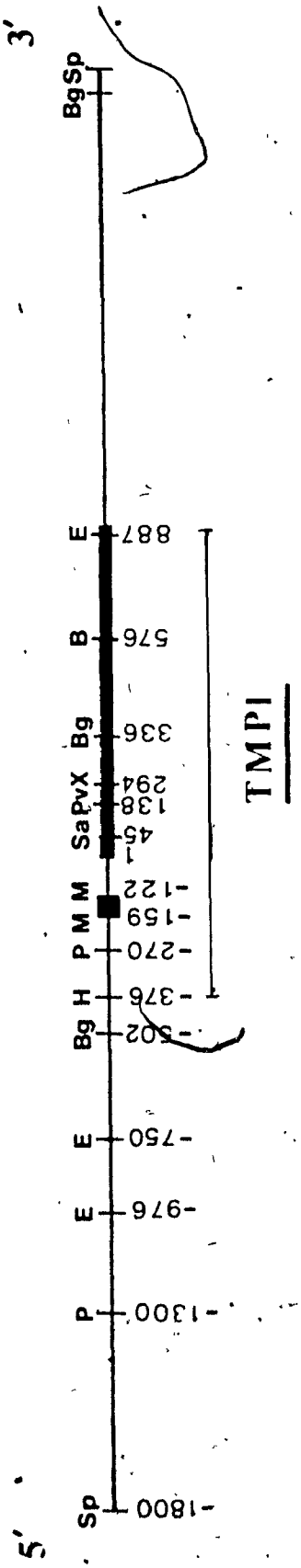
characterization of plasmid DNA by restriction mapping

From this strategy, a total of five independent plasmids were obtained. One plasmid, pRS479 which contained an additional 1.4 kb of DNA, was chosen for further analysis. This analysis generated the partial restriction map of the TMP1 gene and its surrounding region that is shown in figure 2. The restriction recognition sites are indicated above the restriction map while their distances in bps are indicated below relative to the first base of the TMP1 open reading frame. The A of the first codon has been arbitrarily designated as +1.

I wanted to verify that this fragment was indeed the information found upstream of the TMP1 gene. This was accomplished by Southern blot analysis. The arrows in the Southern blot (figure 3) indicate the DNA fragments from pRS479 plasmid DNA and AH22 genomic DNA which hybridized with the 1246 bp HindIII to EcoRI TMP1 probe. The other bands are residual activity from a previous exposure with a LEU2 probe which was not completely removed (data not shown). This blot shows that the plasmid DNA and genomic DNA digested by BglII or EcoRI generated fragments which are the same sizes. These enzymes were chosen since restriction mapping of pRS479 showed that they would generate fragments of known sizes which included sequences from both the retrieved upstream information and the originally cloned DNA sequences (see figure 2). As a molecular weight control, SphI generated a 9.2 kb fragment from pRS479 (figure 3, lane 3), and a 4 kb fragment from AH22 (figure 3, lane 4).

FIGURE 2

Partial restriction map of the TMP1 gene and its flanking region. Distances are indicated in base pairs. The bar (—) beneath the restriction map is the DNA fragment used as the hybridization probe (see text). The restriction recognition sites are indicated as follows: HindIII (abbreviated by H), BamHI (B), SphI (Sp), PstI (P), BglII (Bg), PvuII (Pv), MluI (M), EcoRI (E), and XhoII (X).


3'
BgSp
E
B
Bg
X
Pv
Sa
M
M
P
H
Bg
E
E
P
Sp
887
576
336
294
138
45
1
-122
-159
-270
-376
-502
-750
-976
-1300
-1800
TMPI
5'
Chr XV

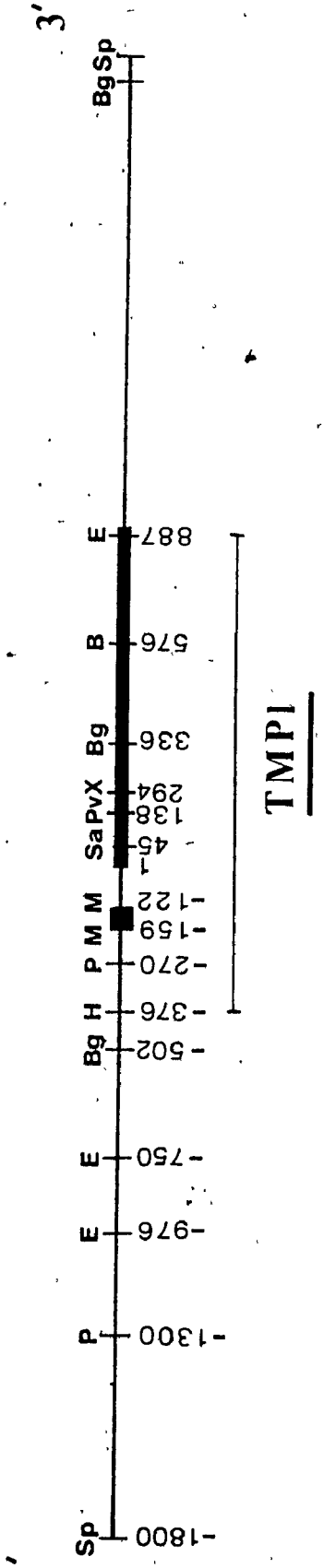

3'
BgSp
E 887
B 576
Bg 336
SaPvX 294
138
45
M 122
M 159
P 270
H 376
Bg 502
E 750
E 976
P 1300
Sp 1800
5'
TMP1
Chr XV

FIGURE 3

Comparison of the restriction map of the cloned TMP1 region with the restriction map of the genomic TMP1 region as determined by Southern analysis. 10 μg of chromosomal DNA from AH22, and approximately 7 X 10^{-3} μg of plasmid DNA from pRS479 were loaded into alternating slots of an 0.8% agarose gel. The lanes contain the following: undigested DNA (lanes 1 and 2), SphI digested DNA (lanes 3 and 4), EcoRI digested DNA (lanes 5 and 6), and BglII digested DNA (lanes 7 and 8). Lanes 1, 3, 5, and 7 contain AH22 DNA, while lanes 2, 4, 6, and 8 are pRS479 DNA. DNA fractionated by electrophoresis in agarose was transferred onto a Pall membrane before probing with the 1246 bp HindIII to EcoRI TMP1 fragment that was radioactively labeled by nick-translation in the presence of [α-^{32}P]dCTP to a specific activity of 5 X 10^6 cpm/μg. The probed membrane was used to expose X-ray film for 3 days. The arrows indicate the bands which hybridize with the TMP1 probe (see text).


1 2
3 4
5 6
7 8

To summarize, this analysis generated the following results which strongly suggested that the 1.4 kb of retrieved DNA is indeed the sequence upstream of the chromosomal TMP1 gene: (i) Southern analysis of the M12B transformants revealed that pRS479 was retrieved from yeast genomic DNA containing plasmid pRS464 integrated at the TMP1 locus (data not shown). (ii) Restriction mapping of the retrieved information present in pRS479 and the Southern mapping of the genomic DNA present upstream of the TMP1 locus resulted in identical maps.

II. MAPPING THE CIS-ACTING SEQUENCES INVOLVED IN TMP1 EXPRESSION

Once I had cloned the additional 1.4 kb of TMP1 upstream information, I was ready to begin characterization of the regulatory information which controls TMP1 expression. To do this, I chose to place the expression of the E. coli lacZ gene under the transcription and translation initiation control signals supplied by sequences from the TMP1 gene. The resulting genes are hybrid genes containing both TMP1 and lacZ sequences. Therefore, I could characterize the role of TMP1 regulatory sequences by determining the levels of β-galactosidase activity in the yeast transformants harbouring TMP1'-'lacZ fusion genes.

II.a. Gene Fusions With Varying Amounts of the 5' Flanking Regions

To locate the cis-acting upstream regulatory sequences, varying portions of this region were subcloned into the

autonomously replicating plasmids, pRS535 and pRS269 (construction discussed by R. W. Ord, PhD thesis, 1987). Briefly, these two plasmids contain the identical 376 bp TMP1 sequences upstream of the TMP1 translation start codon plus 15 codons (pRS535) or 112 codons (pRS269) of the TMP1 N-terminal coding information spliced in frame to the lacZ gene.

II.a.(i) TMP1'-'lacZ fusion gene constructions

Before portions of the retrieved DNA region could be subcloned into the gene fusion plasmids pRS535 and pRS269, two recombinant plasmids, pRS820 and pRS829, were constructed. These plasmids contained two different TMP1 fragments from pRS479 subcloned into the polylinker site of pUC18 and pUC19, respectively (figure 4). pRS829 contains the 2376 bp TMP1 fragment which extends from the SphI site at -1800 bp to the BamHI site at +576 bp. pRS820 contains TMP1 sequences from the PstI site at approximately -1300 bp to the PstI site at -270 bp.

Three additional gene fusion plasmids were then constructed as described in figure 5. These plasmids included the pRS269 derivatives, pRS840 and pRS822, which contained the TMP1 upstream information extending to -1800 bp and -1300 bp, respectively, and a third plasmid, pRS1034, which is a pRS535 derivative containing the TMP1 upstream information extended to about -1300 bp. Once constructed, these plasmids pRS1034, pRS822, and pRS840, were transformed into yeast strain AH22 by the LiCl technique to investigate

FIGURE 4

Shown is the strategy used for constructing pRS820 and pRS829. 3 μg of plasmid pRS479 DNA and 1 μg of plasmid pUC18 DNA were digested either singly by PstI, or doubly by SphI and BamHI to form the pUC derivatives containing the 1 kb PstI upstream TMP1 fragment (pRS820), or the 2.4 kb SphI to BamHI fragment (pRS829). Plasmid pRS479 was further digested by PvuII during construction of pRS820, or digested by Asp718 while constructing pRS829. The ligation mix was used to transform JM83 and white amp^R colonies were chosen for further characterization by restriction enzyme analysis. The thin line (——) depicts the TMP1 DNA sequence; the dashed line (---) depicts URA3 and pBR322 DNA sequences in pRS479; while the thick line (▬) depicts pUC DNA. The sites for SphI, BamHl PstI, HindIII, SmaI and BamHI/BglII are indicated by Sp, B, P, H, and B/Bg, respectively.

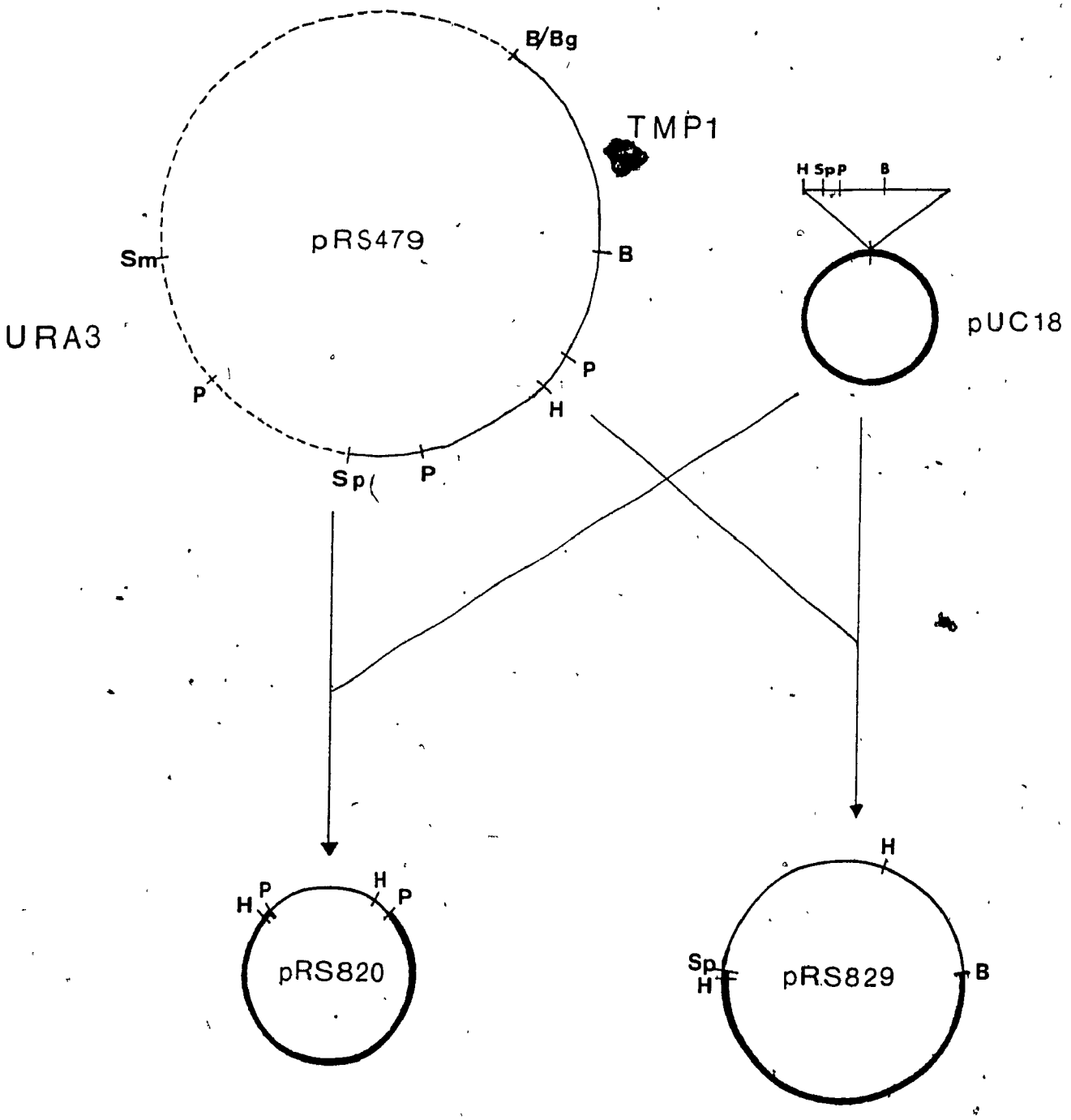
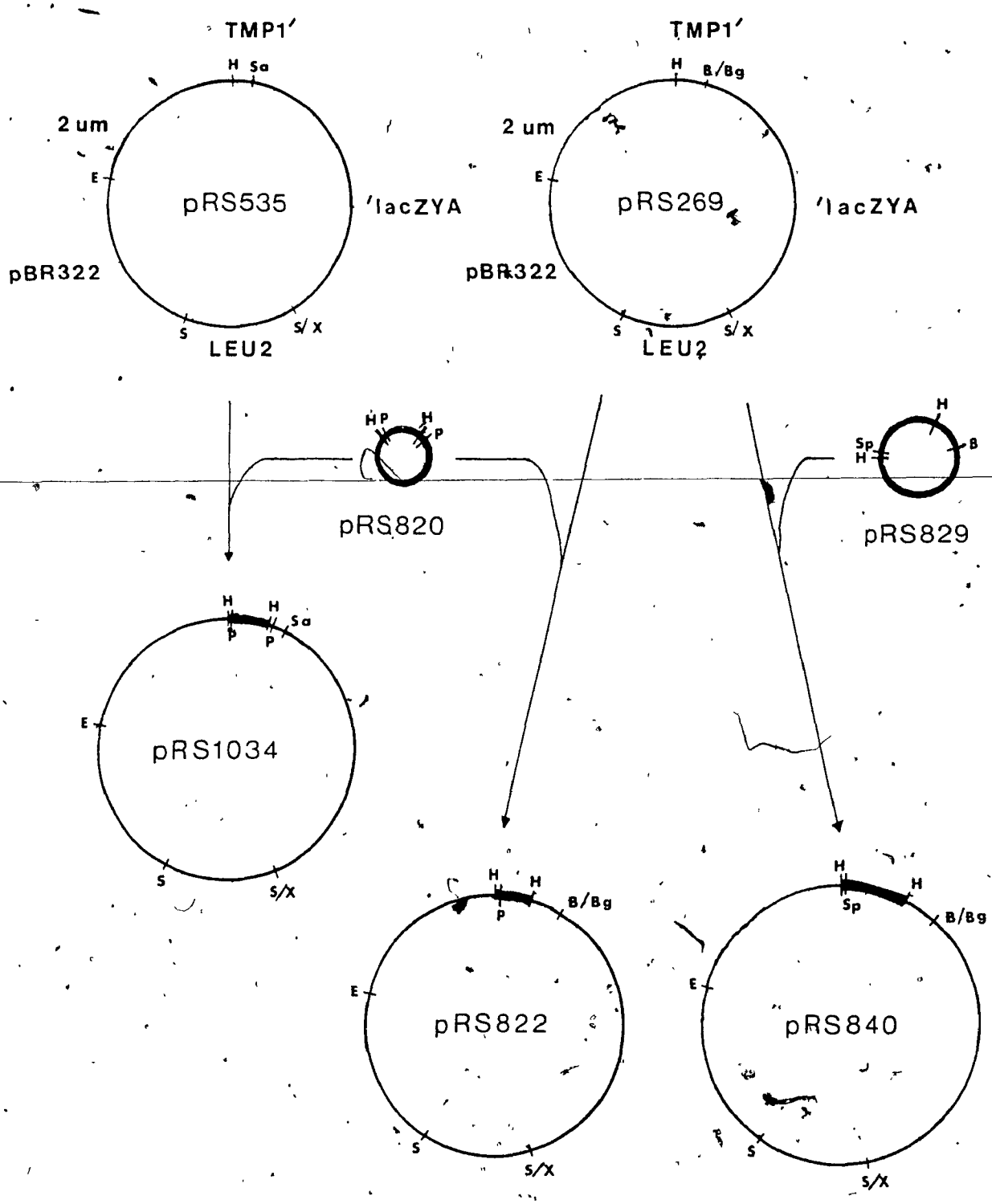

B/Bg
TMP1
pRS479
Sm
B
URA3
P
P
H
Sp
P
H Sp P B
pUC18
H
P
H
P
pRS820
Sp
H
pRS829
B

FIGURE 5

Shown are the partial restriction maps for and the strategy used to construct pRS1034, pRS840, and pRS822. 3 μg of either pRS820 or pRS829, and 1 μg of either pRS535 or pRS269, were digested by HindIII, then ligated using T4 DNA ligase, and used to transform strain JF1754. Colonies that were leu^+ amp^R were chosen for further characterization by restriction enzyme analysis. Shown in A is the construction of pRS1034. The 800 bp HindIII fragment which contained the TMP1 sequence from pRS820 was cloned into pRS535. B shows the construction of the pRS269 derivatives; pRS840 with the upstream information extended to the SphI site, and pRS822 with the upstream information extended to PstI site. The thick line (▬) depicts pRS820 and pRS829 DNA, while the thin line (——) depicts pRS535 or pRS269 DNA. The sites for HindIII, PstI, SphI, SalI, SalI/XhoI, EcoRI, BamHI/BglII, and Sau3a are indicated by H, Ps, Sp, S, S/X, E, B/Bg, and Sa, respectively.


A
B
TMP1′
2 um
pRS535
′lacZYA
pBR322
LEU2
TMP1′
2 um
pRS269
′lacZYA
pBR322
LEU2
pRS820
pRS829
pRS1034
pRS822
pRS840

their expression in yeast. Strain AH22 transformed by autonomously replicating plasmids will be designated as T followed by the plasmid name, e.g. AH22 transformed by pRS1034 was designated by T-1034.

II.a.(ii). TMP1'-'lacZ gene expression in yeast

Once transformants of yeast strain AH22 harbouring each of the plasmids had been obtained, they were grown under selective conditions to mid-log phase and samples harvested to determine their levels of β-galactosidase activity. Also, other transformants obtained from R. Ord (Ph.D., 1987) which contained further deletions and modifications within the TMP1 upstream region were grown and harvested for β-galactosidase determination. A summary of the results obtained with these transformants is shown in table 3. Table 3 gives the levels of β-galactosidase expressed by asynchronous log phase cultures harbouring the different plasmids derived from both pRS535 and pRS269, while figure 6 depicts the partial restriction map of the TMP1 gene showing the relevant portions used in the construction of the different gene fusions. This map is aligned above the TMP1 portions of the different gene fusions given in table 3. Also, the thymidylate synthase activities expressed by AH22 and by a yeast transformant (T-TL1) containing the TMP1 gene on a multicopy plasmid, pTL1, are included in table 3.

A. Expression analysis of pRS535 derivatives

From the levels of β-galactosidase expressed from the different fusion genes derived from pRS535, several

TABLE 3

'lacZ expression from the different gene fusions as indicated

	Strain	Enzyme Activity β-gal[a]	T.S.[b]	Relative levels[c]	Monomer numbers/ cell[d]
1.	T-1034	2.45	nd	1.22	445 (0.01)
2.	T-535	2.01	nd	1.00	365 (0.015)
3.	T-TL30	2.3	nd	1.15	418 (0.017)
4.	T-TL35	11.6	nd	5.8	2106 (0.088)
5.	T-TL31	0.09	nd	0.04	16 (0.00068)
6.	T-744	5.8	nd	2.89	1053 (0.044)
7.	T-741	0.17	nd	0.08	31 (0.013)
8.	T-TL32	0.13	nd	0.06	24 (0.00098)
9.	AH22	nd	26	n/a	2500 (0.10)
10.	T-TL1	nd	252	n/a	24000 (1.00)
11.	T-840	3.46	nd	1.83	628 (0.026)
12.	T-822	2.87	nd	1.52	521 (0.022)
13.	T-269	3.79	nd	2.01	688 (0.027)
14.	T-667	2.91	nd	1.54	528 (0.022)

a. β-galactosidase activity in Miller units:

$$\frac{A_{420}}{\text{min.} \times \text{ml} \times A_{600/\text{ml}}} \times 10^3$$

The levels of β-galactosidase activity expressed by this series of gene fusion transformants are the results from a single experiment. Each β-galactosidase activity value is the mean of duplicate samples which did not vary from the mean by more than 5%. The experiment was performed 4

times and the relative β-galactosidase levels from the different transformants did not vary significantly.

b. specific activity (μU/mg) of thymidylate synthase (courtesy by M. T. Greenwood; pmol ^3H_2O produced/min/mg sol. protein)

c. β-galactosidase expression relative to pRS535

d. β-galactosidase monomer numbers per cell calculated by the formula:

$$\frac{\text{Miller units}}{10^3} \times \frac{4.45 \times 10^{12} \text{ monomers}}{1\ A_{420}/\text{min}} \times \frac{0.24\ A_{600}}{10^7 \text{ cells}} \times 1.7$$

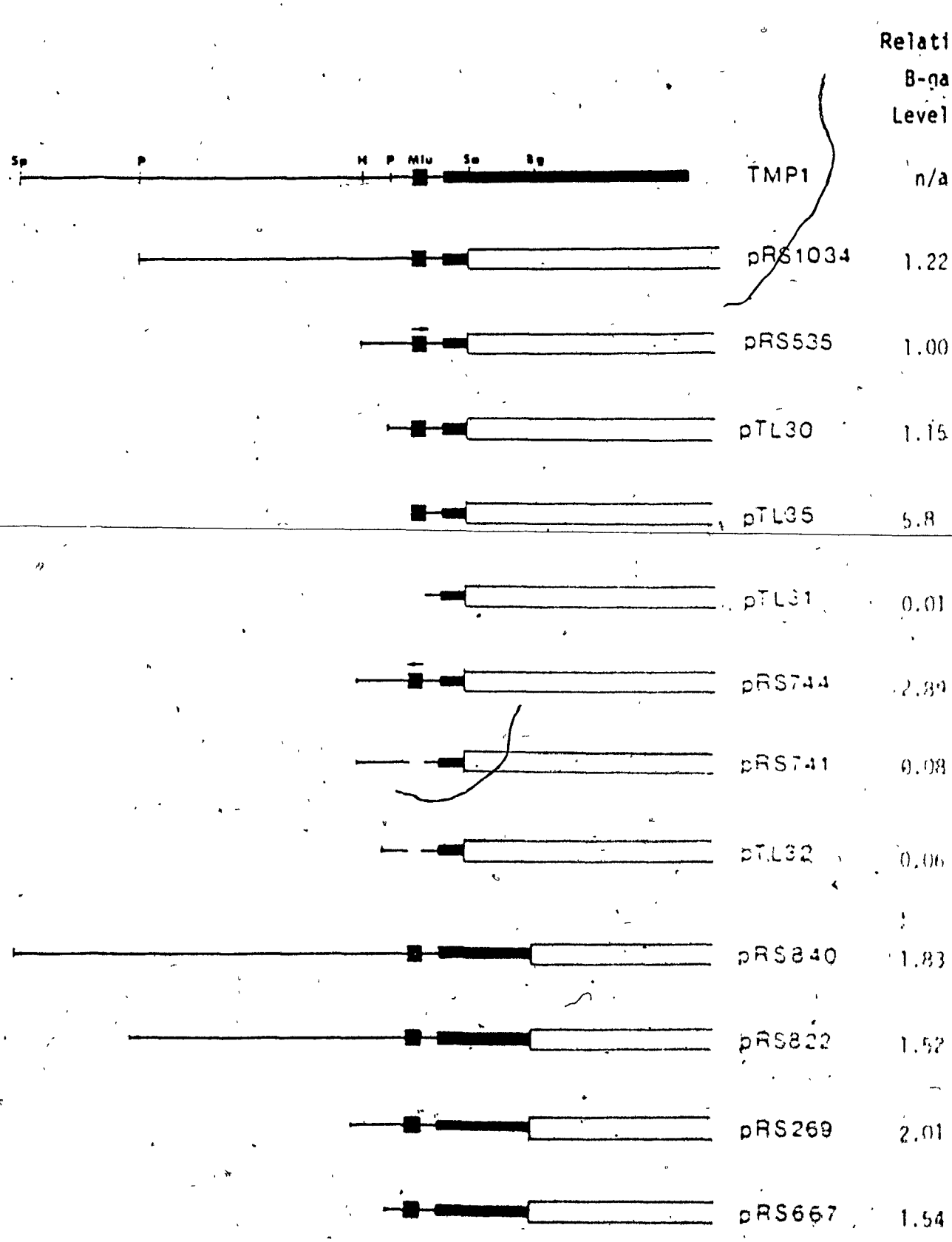
Thymidylate synthase monomer numbers per cell calculated by the formula:

$$\text{spec. act.}_{(\mu U/mg)} \times \frac{0.578 \text{ mg sol protein}}{10^8 \text{ cells}} \times \frac{6.02 \times 10^{11} \text{ molecules}}{\text{pmol } {}^3H_2O}$$

$$\times \frac{\text{min./T.S.}}{73} \times \frac{2 \text{ monomers}}{\text{T.S.}}$$

FIGURE 6

Partial restriction maps show the TMP1 region and the gene fusions having varying portions of the TMP1 upstream information. Thin lined segment (——) depicts the TMP1 upstream sequences while the thick line (▬) depicts the TMP1 open reading frame. The closed box (■) depicts the 34 bp MluI fragment and is indicated by Mlu. The open box (□) depicts lacZ sequence. The TMP1 restriction map shows the relevant restriction recognition sites used in constructing the gene fusion derivatives below. The diagram depicts only the pertinent the TMP1 and lacZ regions of these genes. The plasmid name of the gene fusion is listed at the right of the linear map. The β-galactosidase activity relative to pRS535 is listed at the right of plasmid name. The restriction sites SphI, PstI, HindIII, MluI, Sau3A, and BglII are indicated by Sp, P, H, Mlu, Sa, and Bg, respectively.


Relative
B-gal
Levels
Sp
P
H
P
Mlu
Sa
Bg
TMP1
n/a
pRS1034
1.22
pRS535
1.00
pTL30
1.15
pTL35
5.8
pTL31
0.01
pRS744
2.80
pRS741
0.08
pTL32
0.06
pRS840
1.83
pRS822
1.52
pRS269
2.01
pRS667
1.54

conclusions can be drawn: (i) Information upstream of the PstI site at -270 does not appear to play a role in regulating the levels of TMP1 expression as determined by these experiments. That is, deleting all the information upstream of the PstI site at -270 did not affect the steady state levels of β-galactosidase expression (table 3, lines 1 to 3). (ii) Information between the PstI site at -270 and the MluI site at -159 appears to encode sequences which repress expression. That is, deleting all the upstream information beyond the MluI site at position -159 bp resulted in a 6-fold increase in β-galactosidase expression. (Compare in table 3, line 4 with line 2). (iii) Information between the MluI sites at positions -159 and -122 appears to encode at least part of an upstream activating sequence (UAS). That is, removing all the upstream information beyond position -122 bp resulted in a 22-fold decrease in levels of β-galactosidase expression (compare in table 3, line 5 with 2). Also, when the region between the two MluI sites is deleted to generate the plasmids, pRS741 (table 3, line 7) and pTL32 (table 3, line 8), respectively, the resulting plasmids expressed levels of β-galactosidase which were reduced approximately 15-fold relative to the parental plasmid pRS535. While both plasmids have the 34 bp MluI fragment deleted, the difference between these two plasmids is that pTL32 only has information extended to position -270 bp (figure 6, compare lines 8 and 9). (iv) When the 34 bp MluI fragment was inverted to generate pRS744,

β-galactosidase expression increased 3-fold (table 3, compare line 6 with line 2). This is unexpected result which will be discussed later in the discussion section.

Gene expression from this set of fusion genes suggests that most, if not all, of the upstream information necessary for expressing TMP1 at normal levels during asynchronous growth is located within 270 bp of the start codon. Within this 270 bp region, there appear to be two regions important for regulating TMP1 expression: a negative site located within the positions -270 and -159 bps, and a positive site located within the positions -159 and -122.

B. Expression analysis from the pRS269 derivatives

Different portions of the upstream information were also subcloned into the plasmid pRS269 to form the plasmids pRS840 and pRS822 (figure 6, lines 10 and 11). The gene fusion pRS269 has been shown by M. T. Greenwood to contain information within the TMP1 coding region which affected lacZ expression. The expression from these gene fusions is shown in table 3 (lines 11 to 14). These results also suggested that the retrieved upstream information did not contribute information important for regulating the levels of expression in asynchronous cultures (compare with pRS269 table 3, line 13). All these different gene fusions expressed similar levels of β-galactosidase. In fact, these fusions expressed approximately 1.5 fold more β-galactosidase activity than did pRS535 (table 3, line 2).

Therefore, the gene expression experiments performed

with the different TMP1'-'lacZ plasmids indicated that the retrieved 1.4 kb TMP1 upstream region does not appear to contain information which affected TMP1 expression during asynchronous growth.

II.a.(iii). Estimating the number of monomers of β-galactosidase expressed by the different fusion genes

I was interested in comparing the level at which the different TMP1'-'lacZ fusion genes were expressed with the level at which the native TMP1 gene was expressed. This analysis could be useful for estimating whether the 5' regulatory information identified above encoded all the information necessary to promote normal levels of expression. To carry out this comparison, I chose to calculate the number of monomers of β-galactosidase produced by the different transformants and the number of monomers of thymidylate synthase produced.

Thymidylate synthase monomer numbers were calculated using the specific activity determined by M. T. Greenwood (M. Sc. thesis, 1986), and the turnover number of 73 min^{-1} as determined by Bisson and Thorner (1981). The β-galactosidase monomer numbers were determined using the enzymatic activity determined in this study and that 1 A_{420}/min of purified β-galactosidase corresponds to 4.45 X 10^{12} monomers (Schleif and Wensink, 1981). These terms were used to derive the formula for calculating the number of monomers per cell given in the footnotes for table 3.

Gene expression levels of β-galactosidase encoded by

pRS535 (table 3, line 2) and pRS269 (table 3, line 13) expressed as monomers per cell were compared with the expression levels from the single-genomic copy (table 3, line 9) and from the TMP1 gene when present on the multicopy plasmid pTL1 (strain T-TL1; table 3, line 10). This estimation showed that the number of β-galactosidase monomers expressed by the yeast transformants, T-535 and T-269, was 1.5% and 2.7%, respectively, of the number of thymidylate synthase monomers present in T-TL1. It appears that the β-galactosidase levels expressed from plasmids pRS535 and pRS269 are much lower than would be predicted if they were expressed as efficiently as the TMP1 gene present on pTL1. Also, the number of monomers expressed by T-TL35, containing deletion of the negative control site was only 8.8% of the levels of thymidylate synthase monomers produced by strain T-TL1. Although the lacZ portion of the hybrid genes could affect expression at the transcriptional, translational, or post-translational level, one other possibility is that these low levels of expression are because the TMP1 promoter present on these plasmids is missing information necessary for normal expression (see below).

II.b. An Origin of Replication (ARS) Is Probably Not Involved in Regulating TMP1 expression

Yeast strains which contained TMP1'-'lacZ fusion genes at either the TMP1, or the leu2 locus were constructed. These strains were constructed to determine whether an

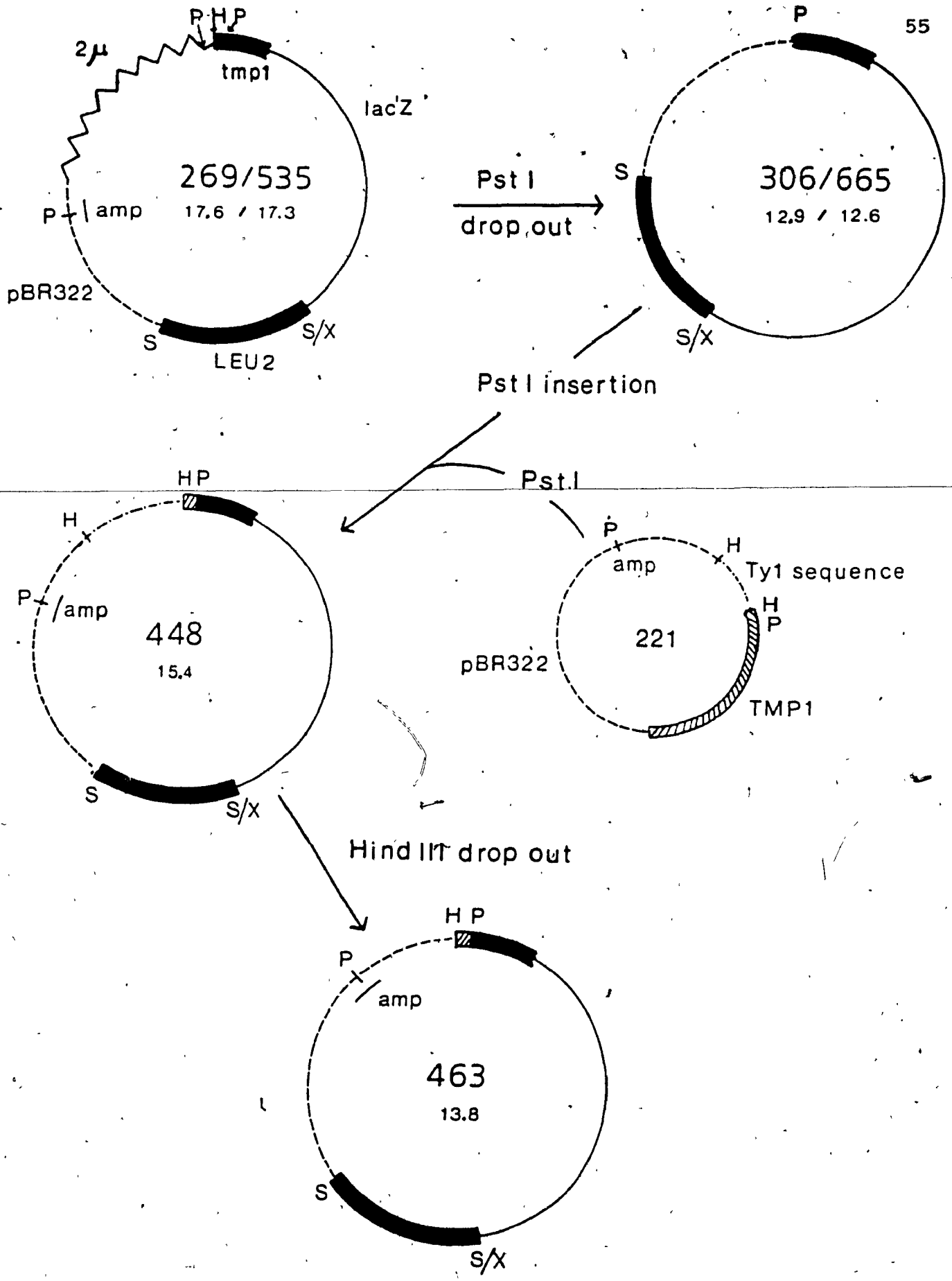
autonomously replicating sequence (ARS) was important for regulating TMP1 expression. Previous results obtained by Hereford and Osley (1982), suggested that an ARS adjacent to the H2B gene was responsible for the periodic transcription of the histone genes H2A and H2B. This was determined by comparing the levels of β-galactosidase expression during the cell cycle from a H2A'-'lacZ gene fusions integrated at the TRT1 locus with the same H2A'-'lacZ gene fusion integrated at the leu2 locus. Thus, to determine if this was also a regulatory feature of TMP1, integrating plasmids carrying the TMP1'-'lacZ gene fusions were constructed and targeted into the yeast genome at either the TMP1 or the leu2 locus of strain AH22. The cell cycle stage dependent expression of β-galactosidase by one of the transformants was determined by M. T. Greenwood (M.Sc., 1986).

II.b.(i). Plasmid constructions

Integrating plasmids were constructed from pRS269 by removing the 2μ information which confers autonomous replication of plasmid sequence in yeast. The construction and structure of the integrating plasmids, pRS306, pRS448, and pRS463 are shown in figure 7. Since these plasmids are derivatives of pRS269, they have 112 amino acids of the N-terminal of thymidylate synthase fused to β-galactosidase. A description of plasmid construction is given in the legend of figure 7.

FIGURE 7

Shown is the strategy used to construct the integrating plasmids pRS306 and pRS463. Plasmid pRS306 was constructed by digesting pRS269 with PstI to drop out 2 μm circle information which resulted in the loss of the 106 bp HindIII to PstI fragment of the TMP1 region and the HindIII to PstI portion of pBR322. The 2.5 kb PstI fragment from pTL221 was cloned into the PstI site of pRS306 to form pRS448. Subsequent digestion by HindIII to delete the yeast Ty sequences resulted in the construction of pRS463. The wavy line (∿∿) depicts the 2 μm circle region; the dashed line (---), pBR322 sequences; the thick line (▬), both tmp1 and LEU2 sequences; the hatched box ([///]), TMP1 sequences in pTL221; the discontinuous line (-·-), Ty sequences; and the thin line (——), lac DNA sequences. The sites for HindIII, PstI, SalI, and SalI/XhoI are indicated by H, P, S, and S/X respectively.


P H P
2μ
tmp1
lac'Z
269/535
17.6 / 17.3
P
amp
pBR322
S
S/X
LEU2
Pst I
drop out
P
S
306/665
12.9 / 12.6
S/X
Pst I insertion
Pst I
HP
H
P
amp
448
15.4
S
S/X
P
H
amp
Ty1 sequence
H
P
221
pBR322
TMP1
Hind III drop out
H P
P
amp
463
13.8
S
S/X

II.b.(ii). Strategy for plasmid integration into the yeast genome

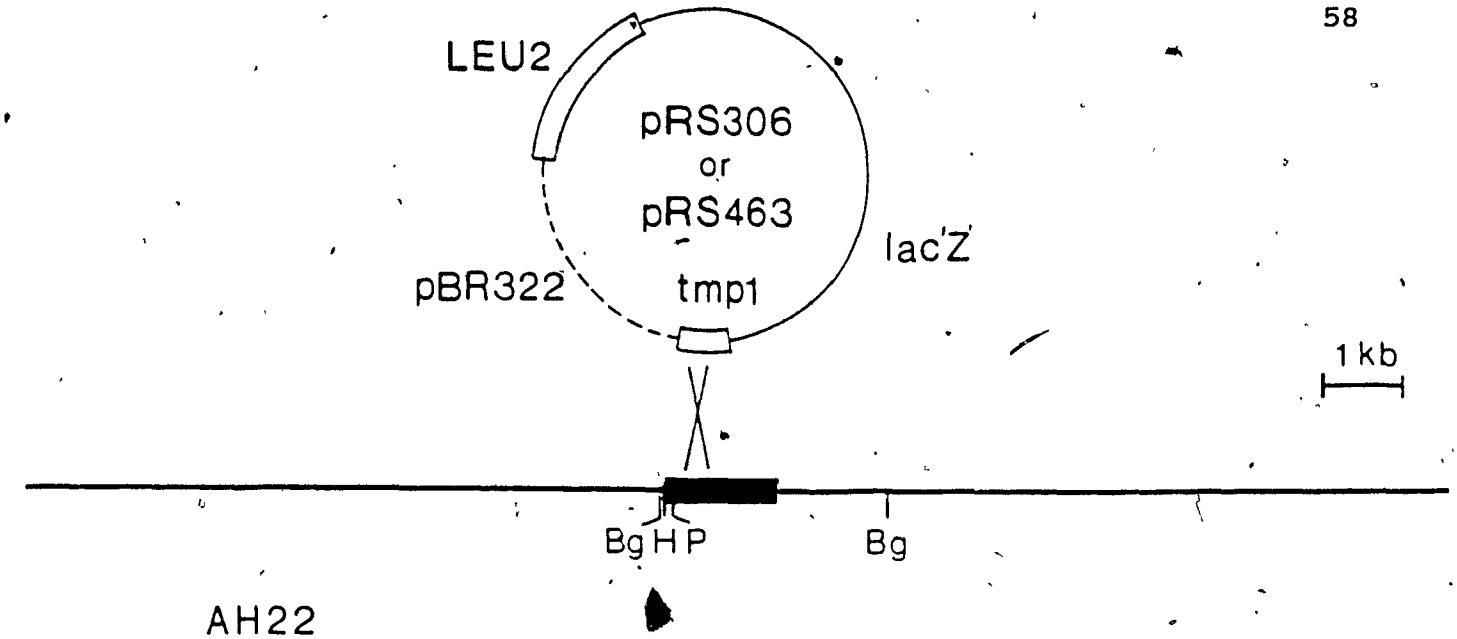
Yeast strain AH22 was made competent by the spheroplast technique rather than the LiCl procedure because plasmid integration is a rare event and a higher transformation frequency can be obtained with this method. Also, a greater transformation frequency is obtained when integrating plasmid DNA has been linearized at a site within the homologous DNA region (Orr-Weaver et al, 1981). This creates recombinogenic ends that facilitate the integration of the entire plasmid into homologous yeast sequence.

No such internal restriction site was available for targeting plasmid sequences to the TMP1 locus. Therefore, pRS306 and pRS463 were linearized by PstI and HindIII, respectively, which digest at the junction between the TMP1 and pBR322 DNA sequences (figure 8). Leucine prototrophs were then selected. Figure 8 shows the predicted chromosomal structure resulting from the integration of a single intact copy of either pRS306 or pRS463 into the TMP1 locus.

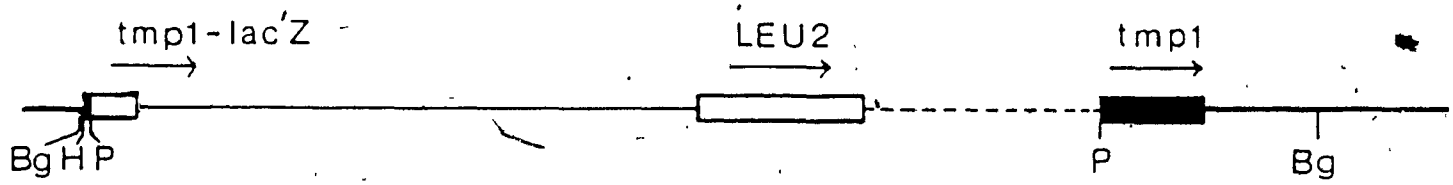
Targeting plasmid sequences to the leu2 locus would be expected to be facilitated by linearizing plasmid DNA with KpnI which cleaves within the LEU2 sequence on pRS306 and pRS463. But, a single intact plasmid copy integrated at leu2 was never obtained. There are two mutations affecting the leu2 gene in AH22 (leu2-3 and leu2-112). If these sites are located on opposite sides of the KpnI restriction cut

FIGURE 8

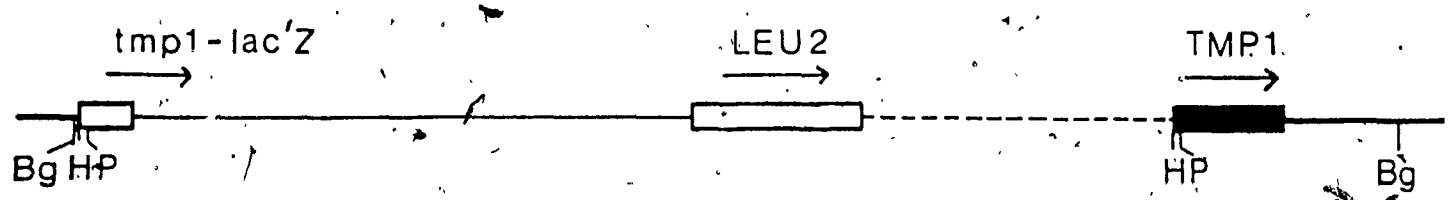
Strategy for targeting integrating plasmids to TMP1 locus. Top line shows pRS306 and pRS463. The line labelled AH22 is a partial restriction map of the TMP1 region in strain AH22. The third and fourth lines depict the partial restriction maps of the TMP1 region after plasmid integration of either pRS306 or pRS463. The open boxes (▭) depict the LEU2 and the TMP1' regions supplied by plasmid sequences. The thin lines (——) depict lac DNA, and the dashed lines (---) depict pBR322 DNA sequences. The flanking region of the TMP1 gene has been depicted as a thick line (▬) and the solid box (■) depicts part of the region of the TMP1 gene that has been cloned. 10 μg of linearized plasmid DNA was targeted to TMP1 genomic site. Plasmids pRS306 and pRS463 were linearized by digestion with PstI and HindIII. Restriction recognition sites for HindIII, BglII, and PstI are indicated by H, Bg, and P, respectively.



AH22



pRS306 integrant



pRS463 integrant

site, a functional LEU2 could only be generated by multiple integration. That is, at least two plasmid integration events must occur to generate a functional LEU2 gene (data not shown). Consequently, the restriction endonuclease SalI which digests at the junction between pBR322 and LEU2 sequences was used to linearize plasmid DNA prior to yeast transformation. Figure 9 shows the predicted structure resulting from the integration of either pRS306 or pRS463 at leu2.

Using the above strategies I obtained three pRS306 and three pRS463 transformants.

II.b.(iii). Analysis of transformants constructed by plasmid integration

Once I had these transformants, I wanted to determine whether the different plasmids had integrated at the TMP1 or the leu2 locus as expected. I also wanted to know the number of plasmid copies which were present in these transformants.

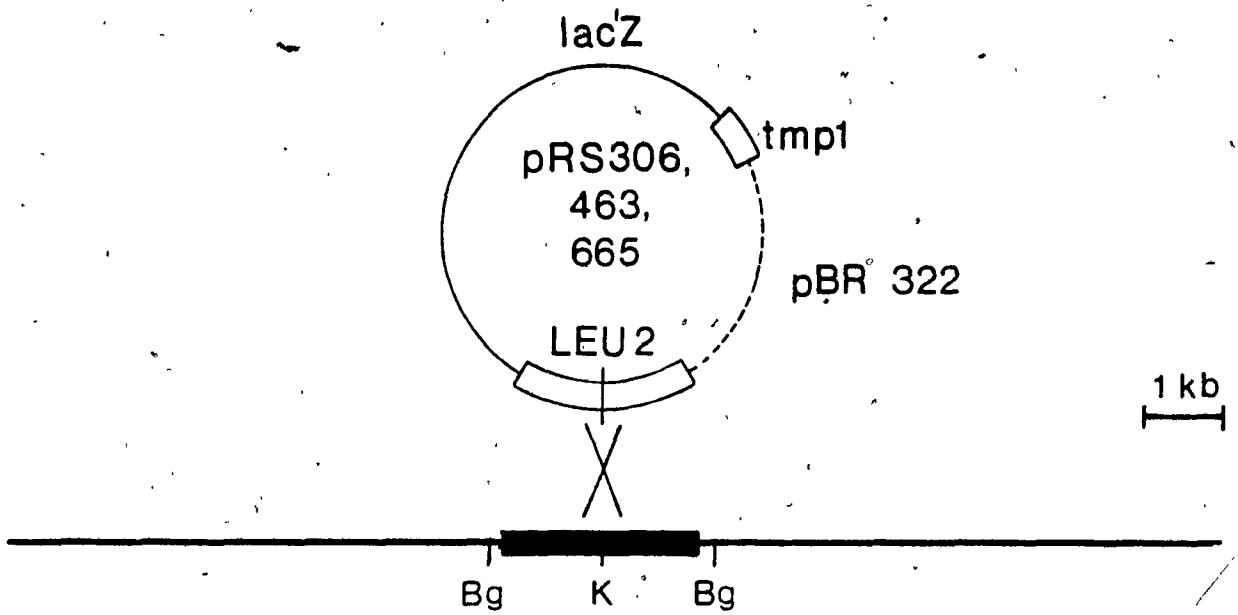
A. Determining the chromosomal site of plasmid integration

To determine the site of integration, BglII digested chromosomal DNA from putative transformants was analyzed by DNA blotting. The blots were then probed with either the TMP1 sequences (954 bp HindIII to BamHI fragment), or LEU2 sequences (2.2 kb SalI to XhoI fragment) which were radioactively labeled by nick-translation.

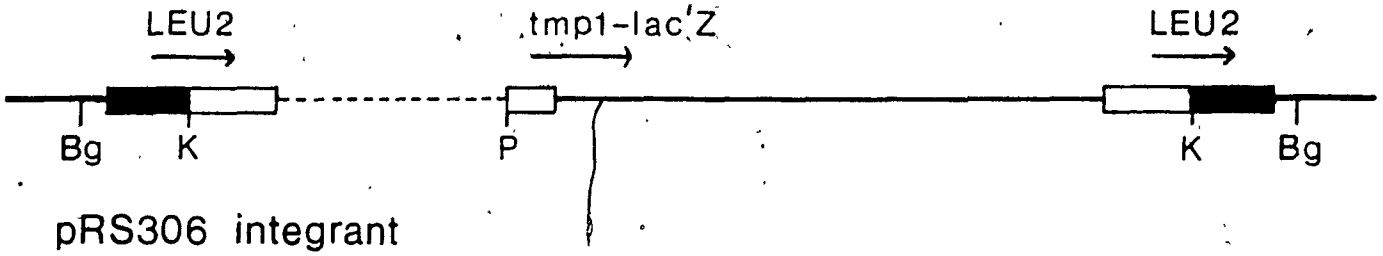
Chromosomal DNA from AH22 digested with BglII contains a 2.75 kb fragment which hybridizes with the LEU2 DNA probe.

FIGURE 9

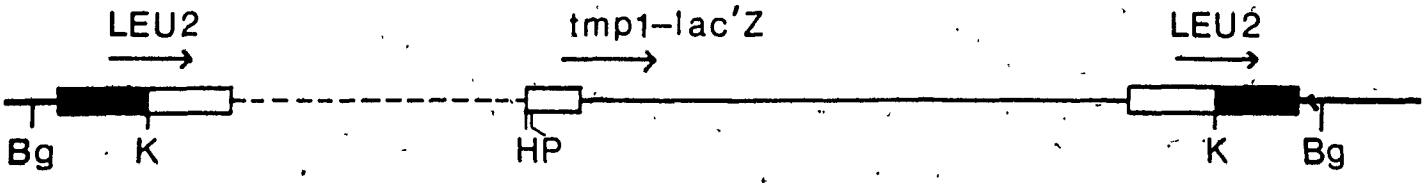
Strategy for targeting integrating plasmids to the leu2 locus. Top line shows pRS306 and pRS463. The line labelled AH22 is a partial restriction map of the leu2 region in strain AH22. The third and fourth lines depict the partial restriction maps of the leu2 region after plasmid integration of either pRS306 or pRS463. The open boxes (▭) depict the LEU2 and the TMP1' regions supplied by plasmid sequences. The thin lines (——) depict lac DNA, and the dashed lines (---) depict pBR322 DNA sequences. The flanking region of the leu2 gene has been depicted as a thick line (▬) and the solid box (■) depicts part of the region of the leu2 gene that has been cloned. 10 μg of linearized plasmid DNA was targeted to leu2 genomic site. Plasmid DNA was linearized by KpnI or SalI before transforming AH22. Restriction recognition sites for HindIII, BglII, and KpnI are indicated by H, Bg, and K, respectively.



AH22



pRS306 integrant



pRS463 integrant

Since neither plasmids (pRS306 and pRS463) contain a BglII cut site, chromosomal DNA that have plasmid sequence integrated at leu2 should have a larger molecular weight BglII fragment that is composed of both genomic and plasmid DNA sequences. This analysis showed that one transformant, RS458, contained plasmid pRS306 integrated at leu2 (figure 10A, lane 12); and two transformants, RS566 and RS567, had plasmid pRS463 integrated at leu2 (figure 10B, lanes 14 and 16).

Similarly, integration at the TMP1 locus would result in the 838 bp BglII fragment of the TMP1 locus (figure 11, lane 2) becoming a larger fragment as a consequence of the integration of plasmid sequences into the chromosomal TMP1 locus (figure 11, lanes 4, 8, and 12). This result showed that transformants RS456 (lane 8) and RS561 (lane 12) contain plasmid pRS306 and pRS463, respectively, integrated at the TMP1 locus.

The site of integration for RS457 (figure 11, lane 10) could not be determined from this blot. However, this strain was still chosen for characterization by Southern mapping (see below) since this strategy could also distinguish whether RS457 had plasmid sequence integrated at TMP1.

FIGURE 10

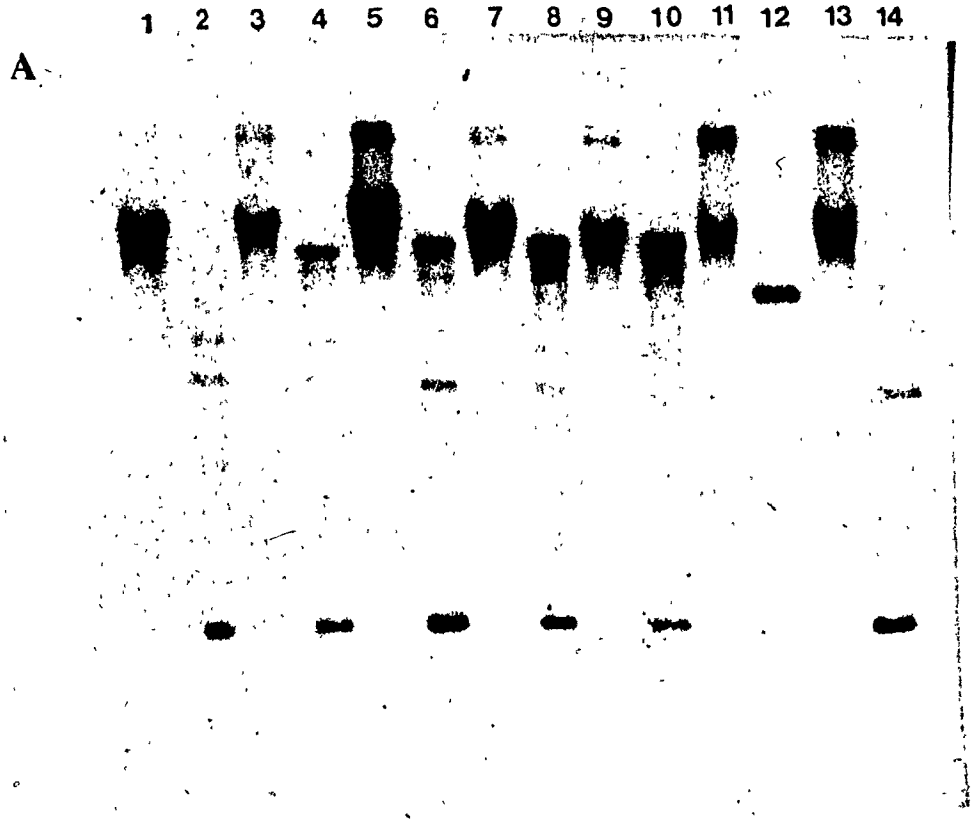
A: Southern analysis of putative leu2 targeted pRS306 transformants

10 μg of chromosomal DNA uncut (odd numbered lanes) or digested by BglII (even numbered lanes) was loaded into alternating slots of a 0.8% agarose gel. The following yeast DNA samples were loaded: AH22 (lanes 1 and 2), RS449 (lanes 3 and 4), RS451 (lanes 5 and 6), RS456 (lanes 7 and 8), RS457 (lanes 9 and 10), RS458 (lanes 11 and 12), and RS414 (lanes 13 and 14). DNA fractionated by electrophoresis was transferred onto a Pall membrane. This blot was probed with the 2.2 kb SalI to XhoI fragment of the LEU2 labelled by nick-translation to the specific activity of 3 X 10^7 cpm/μg in the presence of [α-^{32}P]dGTP. This autoradiograph was exposed for 1 day.

B: Southern analysis of putative leu2 targeted pRS463 transformants

10 μg of chromosomal DNA uncut (odd numbered lanes) or digested by BglII (even numbered lanes) was loaded into alternating slots of a 0.8% agarose gel. The following yeast DNA samples were loaded: AH22 (lanes 1 and 2), RS560 (lanes 3 and 4), RS561 (lanes 5 and 6), RS562 (lanes 7 and 8), RS563 (lanes 9 and 10), RS564 (lanes 11 and 12), RS566 (lanes 13 and 14), and RS567 (lanes 15 and 16). This blot was probed with the 2.2 kb SalI to XhoI LEU2 fragment which was nick-translated to the specific activity of 10^7 cpm/μg

with [α-^{32}P]dGTP. This autoradiograph was exposed for 3 days.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14

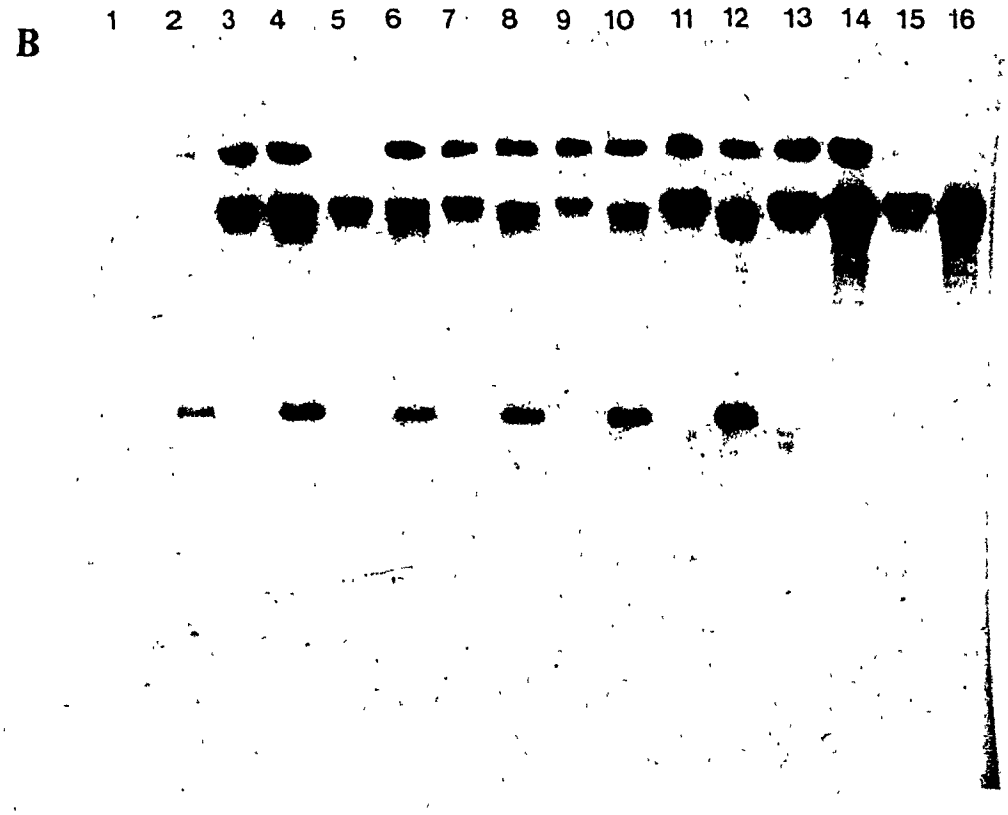
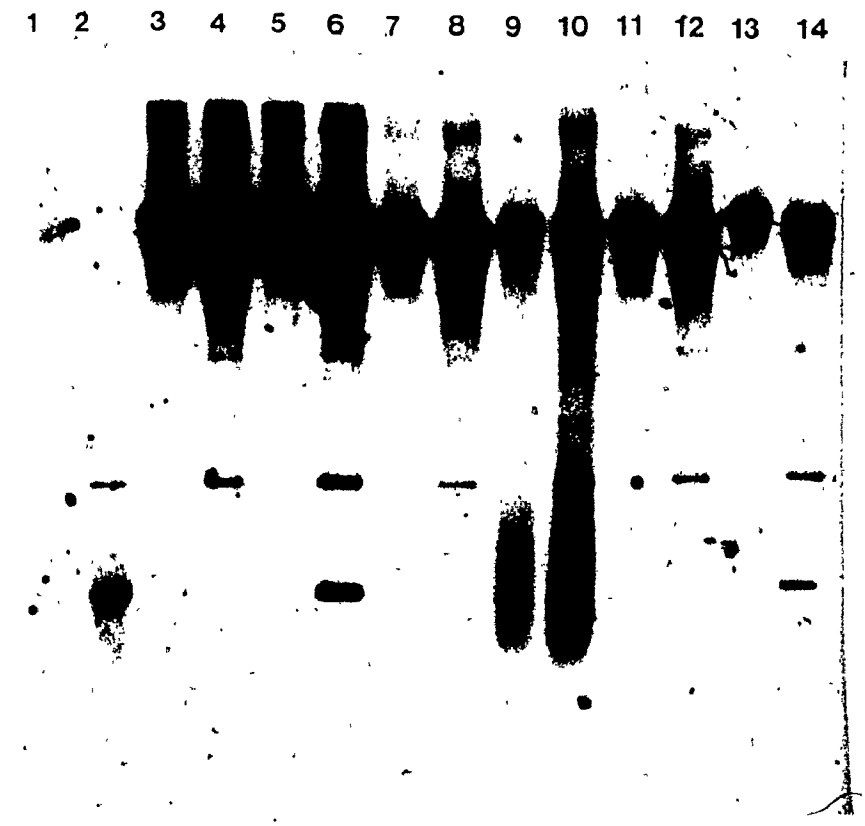

B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIGURE 11

Southern analysis of putative TMP1 targeted transformants. 10 µg of chromosomal DNA was isolated from the following yeast strains: AH22 (lanes 1 and 2), RS452 (lanes 3 and 4), RS453 (lanes 5 and 6), RS456 (lanes 7 and 8), RS457 (lanes 9 and 10), RS561 (lanes 11 and 12), and RS564 (lanes 13 and 14) were loaded undigested (lanes 1, 3, 5, 7, 9, 11, and 13) or digested by BglII (lanes 2, 4, 6, 8, 10, 12, and 14) into the slots of a 0.8% agarose gel. After electrophoresis, DNA was transferred onto Pall membrane. This resulting blot was probed with the 954 bp HindIII to BamHI fragment from the TMP1 gene which had been nick-translated to a specific activity of 4.2×10^6 cpm/µg in the presence of [α-^{32}P]dGTP. This autoradiograph was exposed for 7 days.


1 2 3 4 5 6 7 8 9 10 11 12 13 14

B. Determining the number of plasmid copies at leu2 or TMP1 locus

Southern mapping was performed to determine how many copies of the plasmid had integrated. Single or multiple plasmid integration was distinguished by digesting chromosomal DNA isolated from the different transformants with either SphI, SstI, or KpnI. These enzymes have only one recognition site within the plasmid sequence. Therefore, Southern analysis of a single copy integrant should identify two bands. These two bands should have the combined length which is equal to the sum of the genomic DNA fragment flanked by the chosen restriction sites plus one complete copy of the transforming plasmid. If more than one copy of the transforming plasmid has integrated, three bands are expected: two are the same as the two bands expected from a transformant with a single copy integrated; and the third which would be equal in size to the original plasmid. This strategy distinguishes transformants with one copy from those with two or more copies. However, it cannot distinguish the precise number of plasmids if more than two copies are present. Nevertheless, densitometric scanning of the bands on the autoradiograph was performed and the results used to quantitate plasmid numbers.

The Southern blot of the leu2 integrants RS458, RS566, and RS567, is shown in figure 12A. Since RS458 has two bands (figure 12A, lanes 7 and 11) which have a total length equal to the original genomic DNA plus the length of the

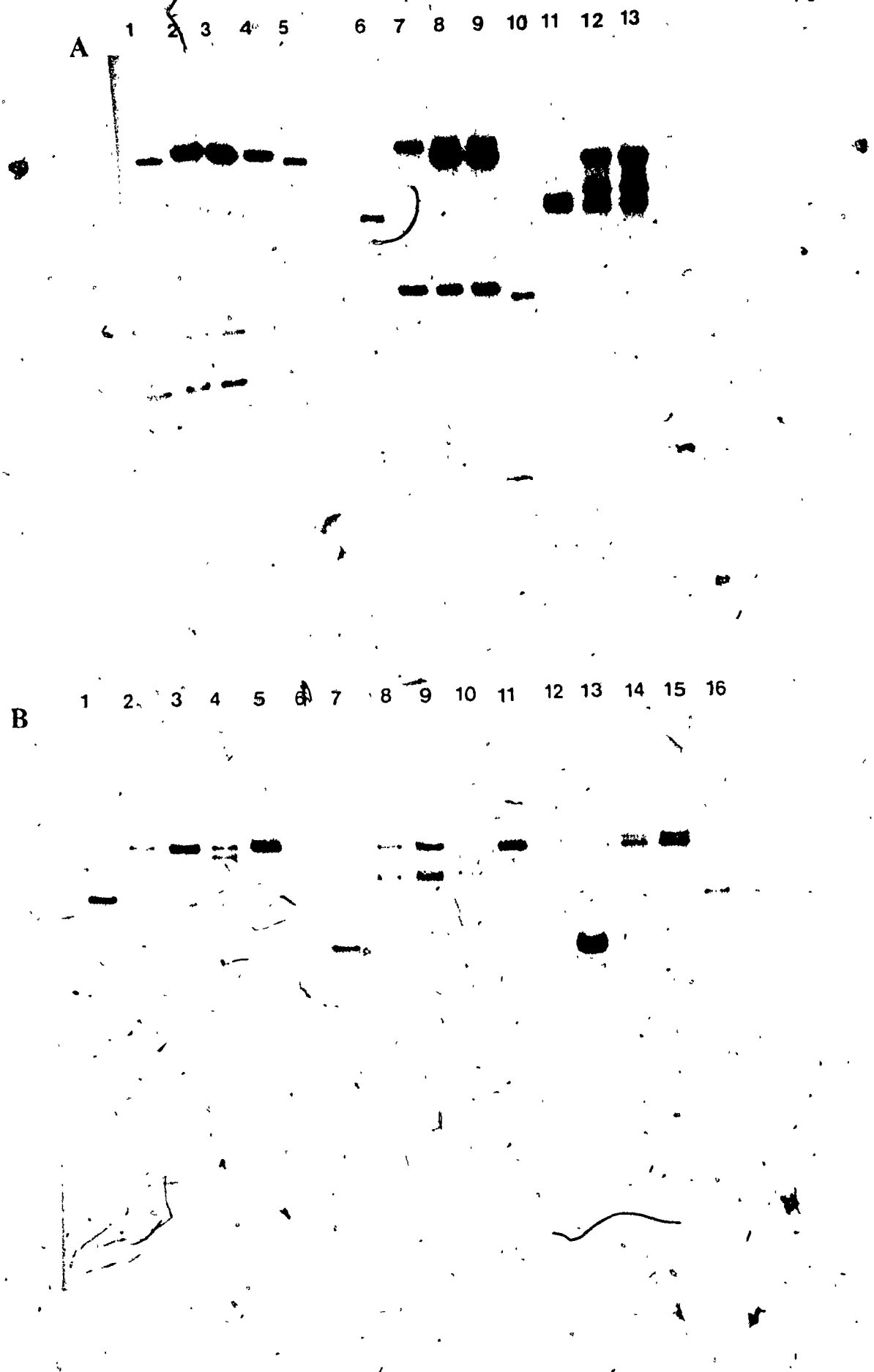
FIGURE 12

A: Determination of the number of plasmid copies integrated at the leu2 locus

10 μg of chromosomal DNA from the yeast strains AH22 (lanes 6 and 10), RS458 (lanes 1, 7, 11), RS566 (lanes 2, 8, 12), and RS567 (lanes 3, 9, 13), was digested by KpnI (lanes 1 to 3), SphI (lanes 6 to 9), or SstI (lanes 10 to 13). Lane 4 contains approximately 0.007 μg of linearized pRS463, while lane 5 has pRS306. This blot was probed with 2.2 kb SalI to XhoI LEU2 fragment which was radioactively labeled by nick-translation in the presence of [α-^{32}P]dATP to a specific activity of 10^7 cpm/μg. This autoradiograph was exposed for 3 days.

B: Determination of the number of plasmid copies integrated at the TMP1 locus

5 μg of chromosomal DNA isolated from the yeast strains: AH22 (lanes 1, 7, 13), RS456 (lanes 2, 8, 14), RS457 (lanes 3, 9, 15), and RS561 (lanes 4, 10, 16) was digested by KpnI (lanes 1 to 4), SphI (lanes 7 to 10), or SstI (lanes 13 to 16). Lanes 5 and 11 contain approximately 0.003 μg of linearized pRS306, while lanes 6 and 12 have approximately 0.001 μg of linearized pRS463 DNA. This blot was probed with the 838 bp BglII TMP1 fragment radioactively labeled by nick-translation (specific activity of 8.5 X 10^6 cpm/μg) in the presence of [α-^{32}P]dATP. This autoradiograph was exposed for 1 day.


A
1 2 3 4 5 6 7 8 9 10 11 12 13
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

transforming plasmid, I can conclude that RS458 has one copy of the plasmid pRS306 integrated at the leu2 locus.

On the other hand, when DNA from the transformants RS566 (figure 12A, lanes 8 and 12), and RS567 (figure 12A, lanes 9 and 13) was subjected to Southern analysis a different result was obtained. In each of these lanes a third or extra band the same length as the original plasmid pRS463 (figure 12A, lane 4) was seen. Therefore, these two transformants have more than one copy of pRS463 integrated at the leu2 locus.

Chromosomal DNA digested by KpnI (figure 12A, lanes 1, 2 and 3) was used to estimate the number of plasmid sequences in the strains RS566 and RS567. Since KpnI digests once within the LEU2 gene, three bands are expected. Two bands which are from the genomic leu2 DNA, and the third band which is the length of the transforming plasmid (figure 9). The intensity of the hybridization of the probe to the DNA bands was determined by densitometry and used to estimate plasmid copy numbers. RS458 (lane 1) was used as the standard reference for one copy of plasmid sequence to estimate the number of plasmid copies present in RS566 (lane 2) and RS567 (lane 3). This revealed that RS566 contained approximately five copies of pRS463 integrated at leu2 while RS567 had about four copies.

The number of plasmid copies for the TMP1 integrants RS456, RS457 and RS561 was analyzed by the same strategy described above for the leu2 transformants. The Southern

blot is shown in figure 12B. This analysis indicated that RS456 (figure 12B, lanes 8 and 14), and from RS457 (figure 12B, lanes 9 and 15) have two copies of plasmid pRS306 integrated at the TMP1 locus. However, in the lanes containing the KpnI digested chromosomal DNA, two bands carrying the TMP1 sequences are seen from RR456 (figure 12B, lane 2) and from RS457 (figure 12B, lane 3). But, restriction mapping analysis revealed that the upper band consisted of two DNA fragments that were not resolved during electrophoresis. Therefore, there are three different DNA fragments from the KpnI digested DNA and these transformants contain two copies of plasmid pRS306 at the TMP1 locus.

In the RS561 transformant, two bands containing TMP1 sequences are seen (figure 12B, lanes 4, 10, and 16) suggesting that a single copy of plasmid pRS463 had integrated at the TMP1 locus. However, addition of the two fragments size from any of the different restriction endonuclease digestions do not equal the sum expected if RS561 contained a single plasmid sequence integrated at the TMP1 locus. Characterization by restriction mapping analysis of the Southern blot indicated that a 2 kb region approximately 5 kb downstream of the structural gene had undergone some sort of rearrangement. The type of rearrangement could not be easily resolved using the data from the autoradiograph. That is, neither a deletion nor an inversion event explained the unexpected pattern from the different DNA digestions. Nevertheless, restriction mapping

analysis suggested that RS561 had a single copy of plasmid pRS463 at the TMP1 locus and the structural gene for TMP1 appeared intact. But it is not known whether this downstream rearrangement affects TMP1 expression.

The following is a summary of the information determined from the Southern analysis of the above integrants. There are: two transformants (RS456 and RS457) which have two copies of plasmid pRS306 integrated at the TMP1 locus; one transformant (RS561) which has a single copy of plasmid pRS463 at the TMP1 locus; one transformant (RS458) which has a single copy of plasmid pRS306 integrated at the leu2 locus and; two transformants (RS566 and RS567) which have approximately five and four copies, respectively, of pRS463 integrated at the leu2 locus.

II.b.(iv). TMP1'-'lacZ gene expression from fusions integrated either at the TMP1 or at the leu2 locus

Once the transformants had been characterized by Southern analysis, β-galactosidase expression by these transformants during asynchronous log phase growth was determined. These results are presented in table 4. Several conclusions can be drawn from this data.

First, the expression of the TMP1'-'lacZ genes appears to be affected by their genomic location. That is, the leu2 integrants RS566 (5 copies of pRS463) and RS567 (4 copies) expressed similar levels of β-galactosidase as the TMP1 integrant RS561 which contains a single copy of pRS463 (table 4).

Table 4

β-galactosidase levels from integrants

Strain	Plasmid (copy numbers)	Site of integration	Relative transformation frequency[a]	Enzyme Activity β-gal[b]	Activity T.S.[c]
1. RS456	pRS306 (2)	TMP1	0.002	0.259	1.5
2. RS457	pRS306 (2)	TMP1	0.002	0.242	nd[d]
3. RS458	pRS306 (1)	leu2	0.002	0.047	5.2
4. RS561	pRS463 (1)	TMP1	0.001	0.145	4.8
5. RS566	pRS463 (>2)	leu2	0.001	0.140	5.2
6. RS567	pRS463 (>2)	leu2	0.001	0.095	nd
7. AH22	n/a[e]	n/a	n/a	0.002	5.9
8. T-269	pRS269	n/a	1.00	2.64	nd

a. Proportion of leucine prototrophs obtained per microgram of closed circular plasmid DNA relative to that obtained with pRS269 (1216 prototrophs/μg DNA).

b. Experiment was repeated twice before determining the average level of β-galactosidase.

c. Thymidylate synthase (T. S.) activity expressed as pmoles of ^3H_2O produced/min/10^8 log phase cells (courtesy of P. P. Poon).

d. not determined

e. not applicable

Second, the information necessary for normal levels of TMP1 expression appears to lie downstream of the HindIII restriction site at position -376, but extends beyond the PstI recognition site at -270. This was concluded from the following analysis. Integration of pRS306 should disrupt TMP1 upstream region at the PstI site. The transformant RS456 integrant which has pRS306 integrated at TMP1 expressed levels of thymidylate synthase levels (courtesy of P. P. Poon) that were 1/4 those obtained with the parental strain AH22 or with RS561 which is a pRS463 transformant and should have the TMP1 locus disrupted at the HindIII site (figure 8, table 4).

Third, there is no ars element associated with the TMP1 sequences used in this study, since the transformation frequencies obtained with all plasmids lacking 2μm circle sequences were very low (table 4), and the integrated plasmid sequences were stably maintained during nonselective growth (data not shown). If there was an ars element or any sequences that could confer autonomous replication, these plasmids would behave like pRS269 which transformed AH22 at a 1000-fold higher frequency and which was rapidly lost under nonselective growth.

II.c. lacZ Expression Using Hybrid Promoters

The gene fusion experiments described above indicated that the 34 bp region between the MluI sites at -159 and -122 was important for the activation of TMP1 expression (table 3). This suggested that this region may contain an

upstream activating sequence for TMP1 expression. Furthermore, the results obtained by M. T. Greenwood (M.Sc., 1986) and R. W. Ord (Ph.D., 1987) indicated that the region controlling periodic expression of the TMP1 transcript is found between the PstI (-270) site and the start codon. Since UAS elements can confer their regulatory properties to other genes, I wanted to determine if this region contained a UAS. Therefore, I constructed hybrid promoter fusion plasmids which replaced the upstream activating sequences, UAS1 and UAS2 (or UAS_C), of the non-cell-cycle-regulated gene fusion, CYC1'-'lacZ (Guarente and Ptashne, 1982), with the AluI fragment from upstream of the TMP1 gene. This AluI fragment contains the sequence between the positions -265 and -90 bps (figure 13, TMP1).

II.c.(i). Plasmid construction

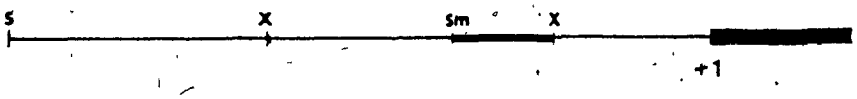
I used two plasmids, pRS845 and pRS2043, to construct the three hybrid promoter plasmids. Plasmid pRS2101 which lacks any upstream activating sequences; plasmid pRS2102 which has the UAS_C replaced by the TMP1 AluI fragment and, plasmid pRS2103 which has the AluI fragment in the inverted orientation. Figure 13 shows the structure and partial restriction map of the upstream regions from these hybrid promoters.

II.c.(ii). Expression analysis

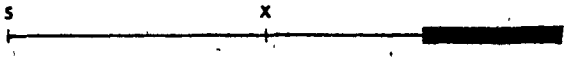
These autonomously replicating plasmids were transformed into strain AH22 by the LiCl technique. The β-

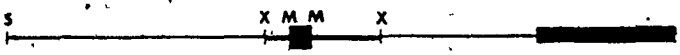
FIGURE 13

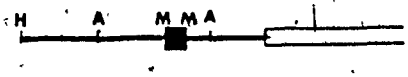
Partial restriction maps of the upstream region of pRS845, the hybrid promoter gene fusions pRS2101, pRS2102, and pRS2103, and the TMP1 gene. The linear maps have been aligned at the A (+1) of the start codon of the open reading frames. The thick line (▬) flanked by Sm and X depicts the CYC1 UAS, and the closed box (■) depicts the MluI fragment. The thin line (——) depicts the 5' flanking region upstream of the CYC1 or TMP1 coding region. The black rectangle (▮) depicts lacZ coding region, while the open rectangle (▭) depicts TMP1 coding region. The plasmids' name are listed to the right. The β-galactosidase activity relative to pRS2101 is listed to the right of plasmid name. The restriction sites SalI, XhoI, SmaI, MluI, HindIII, and AluI, are indicated by S, X, Sm, M, H, and A, respectively.



	Relative B-gal Levels
*p*RS845	94.52
*p*RS2101	1.00
*p*RS2102	0.68
*p*RS2103	0.25
TMP1	n/a







galactosidase activity levels expressed by these transformants during asynchronous growth was determined (table 5). β-galactosidase activity expressed from the TMP1'-'lacZ gene fusions pRS535 and pRS269 has been included for comparison.

lacZ expression from the CYC1 promoter and from the CYC1 promoter deleted for the UAS_C behaved as expected. While pRS845 and pLG669-Z (Guarente and Ptashne, 1982) expressed very high β-galactosidase levels, pRS2101 which has the UAS_C deleted expressed β-galactosidase about 1/100th the levels obtained with pRS845 (table 5). However, in strain AH22, pRS845 expressed 4 to 5-fold more β-galactosidase, approximately 0.9% of total soluble proteins, than was produced by the strain BWG1-7a transformed with pLG669-Z. The reason for this difference is unknown.

Substitution of the UAS_C by the TMP1 AluI fragment resulted in a plasmid (pRS2102) which expressed β-galactosidase at 68% of the level obtained from pRS2101 which is deleted for the UAS (table 5). This result suggested that the TMP1 AluI fragment does not encode any UAS activity. However, pRS2102 expressed 2- to 4-fold more β-galactosidase activity than did pRS535 or pRS269 (table 5, lines 5 and 6, respectively). But the level of β-galactosidase expressed by pRS2102 corresponds to only 5% the levels of thymidylate synthase monomers expressed by pTL1 (table 5, compare line 3 with line 8). This suggested that either the CYC1 upstream region between SalI and XhoI

Table 5

β-galactosidase levels from hybrid promoter fusion plasmids

Strain	Enzyme activity β-gal[a]	T.S.	Relative levels	Monomer numbers/ cell
1. T-845	890.4	nd[b]	94.52	162000 (6.75)
2. T-2101	9.42	nd	1.00	1710 (0.07)
3. T-2102	6.39	nd	0.68	1160 (0.05)
4. T-2103	2.32	nd	0.25	421 (0.018)
5. T-535	1.6	nd	-0.17	290 (0.012)
6. T-269	3.79	nd	0.40	688 (0.019)
7. AH22	nd	26	n/a[c]	2500 (0.010)
8. T-TL1	nd	252	n/a	24000 (1.00)

a. The levels of β-galactosidase activity expressed by this series of gene fusion transformants are the results from a single experiment. Each β-galactosidase activity value is the mean of duplicate samples which did not vary from the mean by more than 5%. The experiment was performed 3 times and the relative β-galactosidase levels from the different transformants did not vary significantly.

b. nd. not determined

c. n/a. not applicable

has information that affected TMP1 expression or the TMP1 AluI fragment contains a weak UAS.

Plasmid pRS2103 which has the AluI fragment in the opposite orientation expressed lacZ at 1/3rd the levels of β-galactosidase expressed by pRS2102 (table 5). This difference may be due to the existence of both positive and negative acting elements within the AluI region. This argument will be discussed later in the discussion.

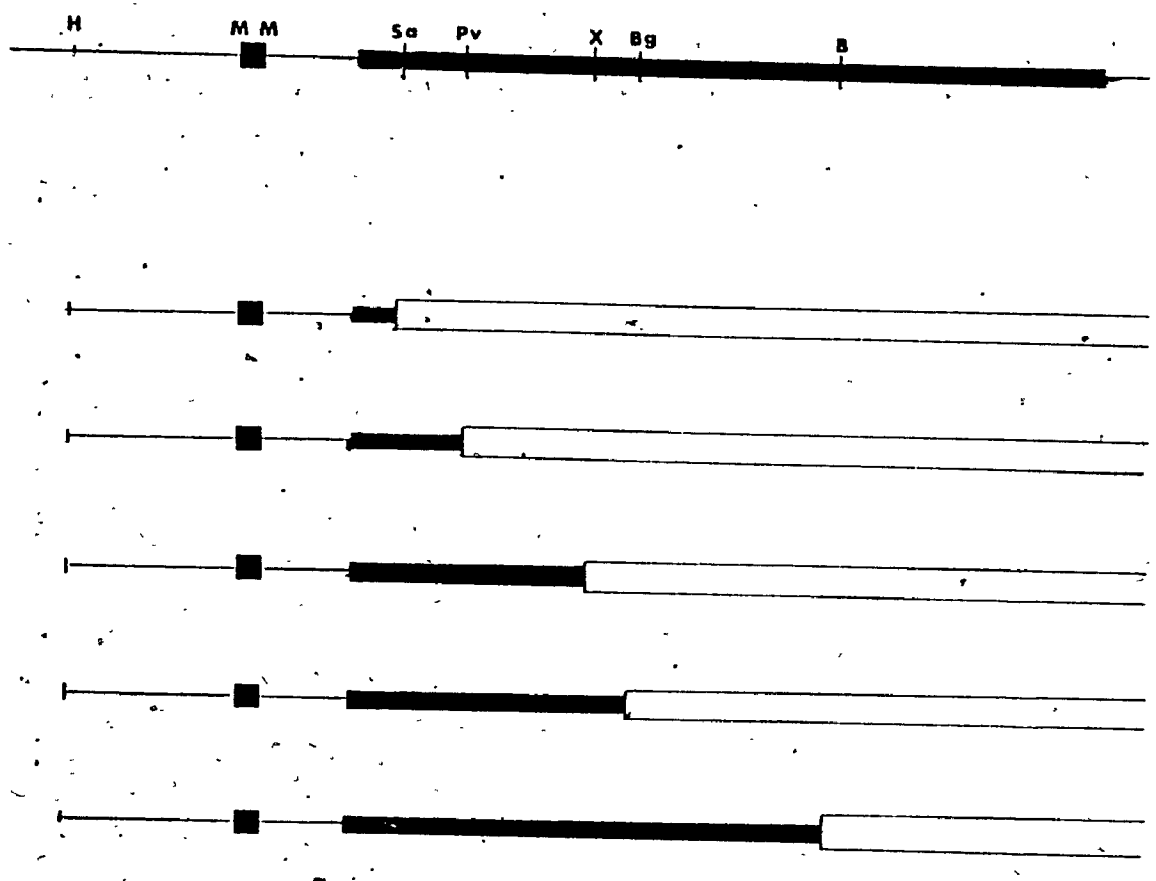
III. ANALYSIS OF THE CODING REGION FOR ELEMENTS AFFECTING EXPRESSION

The above results suggested that the TMP1 upstream region extended out to position -1800 bp could not express lacZ at levels similar to the levels expressed by the TMP1 gene (table 3). One possible explanation is that additional information important for expression is found within the TMP1 structural region. In fact, M. T. Greenwood found that increasing the portion of the TMP1 N-terminal coding region present in the gene fusions increased the level of β-galactosidase expression. A detailed description of these plasmid constructions have been presented elsewhere (M. T. Greenwood, M.Sc., 1986). The pertinent portion of these plasmids is depicted in figure 14. As shown, this set of gene fusions contain upstream information extending to -376 bp and increasing portions of the N-terminal coding region.

To extend these results, I compared the β-galactosidase expression from the TMP1'-'lacZ gene fusions with the expression from the chromosomal TMP1 gene and from the

Figure 14

Partial restriction maps of the gene fusions with varying N-terminal coding regions are shown. The thin line (——) depicts the 5' flanking region of TMP1. The thick line segment flanked by M depicts the MluI fragment. The other thick lined regions (▬) depict the TMP1 coding region. The open box (▭) depicts lacZ sequences. The restriction recognition sites for HindIII, MluI, Sau3A, PvuII, XhoII, BglII, and BamHI are indicated by H, M, Sa, Pv, X, Bg, and B.


H
M M
Sa
Pv
X
Bg
B
TMP1
pRS535
pRS954
pRS852
pRS269
pRS759

plasmid borne TMP1 gene present on the multicopy plasmid pTL1. This comparison was performed by estimating the number of monomers per cell of thymidylate synthase and β-galactosidase (table 6). These estimations indicated that the largest gene fusion, T-759 (table 6, line 5) produced approximately 32% the levels expressed by the plasmid borne TMP1 gene (table 6, line 8). It therefore seems that most, if not all, of the information necessary for expressing TMP1 at normal levels is found between the positions -270 and +564 bp. In addition, this analysis suggests that the region between the 112th and 188th codons encodes information important for normal levels of TMP1 expression.

IV. POST-TRANSLATIONAL REGULATION OF THYMIDYLATE SYNTHASE

Previous results by M. T. Greenwood (1986) suggested that inactivation of thymidylate synthase occurred as cultures of yeast entered stationary phase. He also found that the hybrid thymidylate synthase - β-galactosidase proteins encoded by pRS269 and pRS759 were unstable. Furthermore, this work showed that treatment of yeast with inhibitors of protein synthesis (cycloheximide) and electron transport (sodium azide or cyanide) prevented the decay of β-galactosidase activity. However, when cultures were treated with only cycloheximide, a decrease in enzyme activity was seen. I now wanted to ask whether this post-translational regulatory mechanism was specific only to late log or quiescent phase cells (e.g. in response to nutrient poor conditions), or was it a general regulatory feature

Table 6

Monomer numbers from the gene fusions that contain increasing portions of the N-terminal coding region

Strain[a]	% TMP1 ORF	Enzyme activity β-gal[b]	T.S.	Monomer numbers/ cell	
1. $T\text{-}535_{15}$	4.9	1.01	nd[c]	183	(0.0076)
2. $T\text{-}954_{46}$	15.1	1.74	nd	316	(0.013)
3. $T\text{-}852_{98}$	32.6	2.28	nd	414	(0.017)
4. $T\text{-}269_{112}$	36.8	2.62	nd	476	(0.020)
5. $T\text{-}759_{188}$[d]	61.8	41.83	nd	7600	(0.320)
6. $T\text{-}535_{15}$[d]	4.9	1.92	nd	349	(0.015)
7. AH22	100.0	nd	26	2500	(0.10)
8. T-TL1	100.0	nd	252	24000	(1.00)

a. subscripts indicate the length of TMP1 N-terminal coding region fused to lacZ

b. The levels of β-galactosidase activity expressed by this series of gene fusion transformants are the results from a single experiment. Each β-galactosidase activity value is the mean of duplicate samples which did not vary from the mean by more than 5%. The experiment was performed 3 times and the relative β-galactosidase levels from the different transformants did not vary significantly.

c. nd. not determined

d. separate experiment which was not repeated

that occurred at all stages of batch culture growth?

Therefore, yeast cultures containing the different gene fusions were grown to early log (average A_{600} 0.036) and midlog (average A_{600} 0.250) before splitting in half for treatment with cycloheximide alone or with both cycloheximide and sodium azide. Aliquots (9 ml) were withdrawn to determine β-galactosidase activity levels at t_0 immediately prior to the inhibitor treatment. Samples were harvested after 6 hours (table 7).

This analysis showed that: (i) TMP1'-'lacZ gene fusions containing less than the first 46 TMP1 N-terminal codons expressed hybrid proteins that were stable following the addition of cycloheximide or both cycloheximide and azide. Also, pYT760-ryp3 (strain T-ryp3), which has a random yeast promoter fused to the lacZ coding region, expressed a protein that was stable during both types of inhibitor treatments. This result is consistent with those previously obtained by M. T. Greenwood. (ii) Gene fusions, having between 46 and 188 N-terminal codons, expressed β-galactosidase activity that were unstable during both types of inhibitor treatment. The fusion proteins with 99 and 112 N-terminal codons decreased 10-fold during the treatment interval while the fusion protein with 188 codons decreased 3-fold. From this result it appears that the inactivation of the hybrid thymidylate synthase - β-galactosidase fusion proteins during the early part of batch culture growth, is energy independent. This contrasts with the results

Table 7

Inactivation of β-galactosidase

Strain[a]	β-galactosidase activity[b] A_{600} 0.035[c]			A_{600} 0.250[c]		
	N.T.[d]	w/CX[e]	w/CX&AZ[e]	N.T.[d]	w/CX[e]	w/CX&AZ[e]
$T\text{-}535_{15}$	1.0	1.0	1.0	2.08	2.02	2.07
$T\text{-}954_{46}$	1.7	1.7	1.7	3.13	3.04	3.04
$T\text{-}852_{98}$	2.3	0.3	0.3	4.08	0.69	0.51
$T\text{-}269_{112}$	2.6	0.12	0.3	7.42	0.8	0.75
$T\text{-}759_{188}$	22.6	7.55	5.25	nd	nd	nd
T-ryp3	4.0	3.8	3.8	6.55	6.26	6.49
AH22	0.02	0.04	0.02	0.08	0.00	0.02

a. subscripts indicate the length of TMP1 N-terminal coding region fused to lacZ

b. The levels of β-galactosidase activity expressed by this series of gene fusion transformants are the results from a single experiment. Each β-galactosidase activity value is the mean of duplicate samples which did not vary from the mean by more than 5%. The experiment was performed 4 times and the relative β-galactosidase levels from the different transformants did not vary significantly.

c. Cultures were grown for 20 hours in YNBDH media (approximately 8 generations) to the indicated A_{600} before harvesting for t_0 sample or treatment by inhibitors.

d. No treatment (N.T.) by inhibitors.

e. The final concentration of 100 μg/ml of cycloheximide (CX) alone or 100 μg/ml of cycloheximide and 100 μg/ml of Na azide (Az) were added to 25 ml aliquot from each culture and samples harvested after 6 hours for β-galactosidase determination.

obtained by M. T. Greenwood who found that these same lacZ fusion proteins were inactivated during cycloheximide treatment but not during treatment with cycloheximide and azide.

To further characterize this apparent discrepancy I did the following experiments. In the first experiment, transformants containing the plasmids pRS535, pRS269, and pYT760-ryp3 were grown to three different batch stages (early, late, and stationary), and then treated with cycloheximide or with cycloheximide and Na azide (table 8). This experiment showed that in the cultures treated when they were at an average A_{600} of 0.030, sodium azide did not prevent the inactivation of β-galactosidase encoded by pRS269 since 90% of the enzyme activity was lost during the 6 hours following addition of inhibitor. However, during late log (average A_{600} of 2) phase, Na azide prevented the loss of β-galactosidase activity which occurred during cycloheximide treatment. The stable fusion proteins expressed from T-535 and T-ryp3 remained at the same levels following the addition of cycloheximide irrespective of batch stage or presence of Na azide. In the second experiment, when transformants containing the plasmids pYT760-ryp3 or pRS269 were sampled at early and late log stages of batch growth, similar results were obtained (table 9). For the third experiment, two different concentrations (5 and 100 μg/ml) of cycloheximide and Na azide were tested to determine whether the high concentration of 100 μg/ml

Table 8

Batch culture stage dependent inactivation of β-galactosidase

Strain	A_{600}[a]	β-galactosidase activity[b] No treatment	w/ CX	w/ CX & AZ
T-535	0.032	1.9	1.6	1.6
T-269	0.018	5.8	0.41	0.75
T-ryp3	0.044	7.0	6.7	6.8
-T-535	0.666	2.25	2.1	2.1
T-269	0.878	4.84	0.5	3.5
T-ryp3	0.954	4.24	4.14	4.15
T-535	1.966	4.0	3.95	3.94
T-269	2.084	4.2	0.70	4.17
T-ryp3	2.202	3.9	3.9	3.9

a. each culture was grown for 20 to 23 hours in YNBDH media (approximately 8 to 10 generations) to reach the indicated A_{600} before harvesting (no treatment sample) or treatment with the inhibitors indicated.

b. The levels of β-galactosidase activity expressed by these gene fusion transformants are the results from a single experiment. Each β-galactosidase activity value is the mean of duplicate samples which did not vary from the mean by more than 5%.

Table 9

Batch culture stage dependent levels of β-galactosidase

Strain	A_{600}[a]	β-galactosidase activity[b] No treatment	w/ CX	w/ CX & AZ
T-ryp3	0.366	5.45	5.55	5.24
T-269	0.350	3.92	0.22	0.47
T-ryp3	1.958	4.36	4.3	3.85
T-269	1.962	4.4	0.36	4.3

a. 25 ml aliquot were sampled from the yeast cultures at the A_{600} indicated.

b. The levels of β-galactosidase activity expressed by these transformants are the results from a single experiment. Each β-galactosidase activity value is the mean of duplicate samples which did not vary from the mean by more than 5%.

inadvertently perturbed cellular metabolism (table 10). This experiment indicated that the instability of the hybrid thymidylate synthase - β-galactosidase protein encoded by pRS269 is not dependent on the concentration of inhibitors. The different concentrations of sodium azide used did not prevent the loss of β-galactosidase during the 6 hours of inhibition.

Together, these experiments showed that at early and midlog phase of batch culture growth, sodium azide did not prevent the inactivation of fusion proteins from T-269. However, it prevented the loss of β-galactosidase activity in late log phase and stationary phase. This indicates there are batch culture stage dependent differences between the mechanisms involved in inactivating β-galactosidase expressed from pRS269.

Table 10

Concentration independence of inactivation

Concentration (μg/ml)[a] CX	AZ	β-galactosidase activity[b]	Relative levels
none	none	2.62	100
100	none	0.12	4.6
5	none	0.12	4.6
100	100	0.30	11.5
100	5	0.09	3.4
5	100	0.29	11.1

a. The final concentration of inhibitors, as indicated, was added to 25 ml aliquot of yeast T-269 culture. Inhibition was carried out for 6 hours before harvesting for β-galactosidase determination.

b. T-269 batch culture was grown for 20 hours (A_{600} 0.095) before harvesting for t_0 sample or treatment by the inhibitors. Each β-galactosidase activity is the mean of duplicate samples which did not vary by more than 5%. This experiment was not repeated.

DISCUSSION

TMP1'-'*lacZ* gene fusions were used to localize the regulatory regions of the *TMP1* gene. Since the *lacZ* portion lacks transcriptional and translational start signals, provision of these signals by the *TMP1* moiety results in the synthesis of thymidylate synthase - β-galactosidase proteins. The *TMP1* portion of these fusion genes was altered by deleting varying portions of either its upstream or N-terminal coding region. This approach was an indirect means of delineating the regulatory regions of the *TMP1* gene. The major findings of this analysis were: (i) At least two *cis*-acting upstream sequences controlling *TMP1* expression are found within 270 bp of the *TMP1* open reading frame. (ii) The *TMP1* gene appears to have a weak promoter. (iii) The promoter region consists of multiple elements that act in concert to regulate *TMP1* expression. (iv) *TMP1* information within the coding region can affect the expression of *TMP1*'-'*lacZ* expression. (v) There appear to be two distinct batch culture stage dependent mechanisms for the inactivation of the hybrid fusion protein encoded by pRS269.

Characterization of the 5' upstream region

My analysis began by isolating a 1.4 kb genomic DNA fragment from upstream of the *TMP1* gene. This fragment extended from the *Hin*dIII site at -376 bp to a *Sph*I site at -1.8 kb. Once this region was retrieved, it was confirmed to be contiguous with the chromosomal *TMP1* gene by

restriction enzyme mapping and by Southern blot analysis (figure 2). I then cloned this 1.4 kb DNA fragment and a 800 bp portion of this fragment immediately upstream of the TMP1'-'lacZ gene fusions present on pRS535 and pRS269 (figure 5). The level of β-galactosidase expressed by these three new fusion genes, with the extended upstream information (-1800 and -1300 bps), was compared with fusion genes which had progressively decreasing amounts of 5' flanking DNA (table 3).

The levels of β-galactosidase expressed from the fusion genes which extended to -1800 or -1300 bps indicated that the retrieved DNA region did not contain information which affected the levels of TMP1 expression during normal logarithmic growth. This suggested that the retrieved region does not have a role in regulating steady state levels in log phase cells. However, it may still have a role in regulating TMP1 expression in response to other physiological signals, such as dTTP levels, cell cycle stage, or growth conditions, which were not tested in this study.

Expression analysis of several other TMP1'-'lacZ fusions derived from pRS535 suggested that the upstream sequences involved in regulating levels of TMP1 expression were found within 270 bp of the TMP1 open reading frame. Within this 270 bp region, there appear to be at least two upstream elements that are important for TMP1 regulation. One element, which is encoded within or overlaps the region

located between the positions -270 and -159, has a negative role since when this region is deleted expression increases 6-fold. The other element, which is located within or overlaps the sequences between positions -159 and -122, has a positive role since when this region is deleted expression is severely reduced (table 3).

Deletion of the same region (-159 to -122 bps) from the TMP1 gene resulted in a decrease in TMP1 RNA levels and altered transcription initiation sites (E. MacIntosh, pers. comm.). This indicates that this region plays a positive role in TMP1 expression. This together with the finding that it is located upstream of the TMP1 transcription initiation sites tentatively identifies this region as part of an upstream activating sequence.

Although the region between -159 and -122 is critical for the positive activation of TMP1 expression, it may also contain part of the recognition sequences for a negative trans-acting factor. First, the plasmid pTL35 which is deleted for all the information upstream of the -159 bp position, expressed 6-fold more β-galactosidase activity than pRS535 (table 3). This suggests there is a negative element upstream of the -159 bp position. Second, inverting the region between -159 and -122 bp resulted in a 3-fold enhancement of β-galactosidase activity. This seems surprising since most UAS elements studied to date are equally efficient in either orientation (Struhl, 1986a). One possible explanation for this discrepancy is that

inverting this fragment does not affect the positive element but does alter a negative element. If this is correct and if the affected element is the same element as the one between -270 and -159 bps then, the negative site overlaps the MluI site at position -159. It therefore appears that a negative element lies upstream of the TMP1 UAS, and may contain part of its recognition sequences within the sequences implicated as encoding the positive regulatory region which is in between positions -159 and -122 bps.

The proximity of the repressor site and our putative UAS suggested a regulatory mechanism which involves competition between the trans-acting negative and positive factors for overlapping sites. Also, plasmid pRS2103 which contains the inverted AluI fragment, expressed 3-fold less β-galactosidase than did pRS2102 which has the AluI fragment in the correct orientation (table 5). This suggested that the mechanism of repression is by steric hindrance when the AluI fragment is in the correct orientation and a combination of steric hindrance and blockage when the AluI fragment is in the opposite orientation. If this interpretation is correct (that is, the repressor site is upstream of the UAS and the repressor acts via a steric hindrance method), it is an inefficient method of repression since repression is 3-fold more effective when the negative site is located downstream between the MluI region and the open reading frame (table 5). However, the TMP1 promoter appears to be a weak promoter (see below); consequently, an

efficient repression system may be unnecessary.

Since this study used gene fusions that were constructed by simple subcloning manipulations, the exact boundaries of the positive and negative cis-acting sequences have not been defined. This can be determined by using the exonucleases Bal31 or ExoIII to delete information from the 270 bp flanking the coding region. This would confirm whether there are two distinct or overlapping regulatory regions, and whether there are other elements not found in this study. Once localized, in vitro mutagenesis (e.g. linker scanning) could be used to verify that the regulatory sequences have been correctly identified. Also, their UAS or URS activity can be studied separately by cloning these regions into a gene such as CYC1, which is expressed at a constant rate throughout the cell cycle, to determine their effect on another gene. For instance, can either or both regions cause the periodic expression of the CYC1 gene?

The TMP1 promoter directs low levels of lacZ expression

The expression of TMP1 and TMP1'-'lacZ genes were compared by estimating the number of thymidylate synthase and β-galactosidase monomers present in log phase cells. This analysis showed that the β-galactosidase levels expressed from pRS535 were much lower than the levels of thymidylate synthase expressed from pTL1 (table 3). Even deletion of the negative control site (pTL35) did not produce levels of β-galactosidase (only 9%) comparable to those of thymidylate synthase (table 3). This suggested

that the TMP1 promoter sequences present on pRS535 did not contain all the information necessary to promote normal levels of TMP1 expression. Together, this suggested that the TMP1 promoter sequence present on pRS535 is inefficient in activating gene expression when compared with TMP1 gene itself. It appears that pRS535 does not contain all the information necessary to elicit normal levels of expression.

Since lacZ is not native to yeast, the presence of this information in hybrid gene fusions could inadvertently affect expression at the transcriptional, translational, or post-translational level. A decision as to whether the TMP1 gene has a weak promoter awaits further analysis.

Preliminary identification of a translational activator within the TMP1 open reading frame

The steady state level of β-galactosidase expression was determined from a set of gene fusions that contained increasing portions of the TMP1 N-terminal coding region (table 6). This experiment indicated that a region encoded at least in part by the sequences between the 112th and 188th codon of the TMP1 open reading frame contained information which enhanced TMP1 expression. Enhancement of expression appears to occur at the post-transcriptional level since deleting the genetic information between the 112th and the 195th codons of the native TMP1 gene did not affect the levels of mRNA expression (E. MacIntosh, pers. comm.).

Downstream activating sequences (DAS) have also been

found in the open reading frames of the glycolytic genes phosphoglycerate kinase (PGK) and pyruvate kinase (PYK). Their studies (Mellor et al, 1987; Purvis et al, 1987) suggested that the DAS of PGK and PYK influences the rate of transcription. Without the DAS, the hybrid mRNA levels were approximately 10-fold less than the native PGK or PYK mRNA levels. When the N-terminal coding region of PGK was extended in the hybrid gene fusion plasmids to include the DAS, hybrid mRNA levels increased to levels comparable to that expressed by PGK. These authors suggest that the DAS of these glycolytic genes is a positive control site that acts at the level of transcription.

The putative TMP1 DAS apparently acts at a post-transcriptional level; possibly at the translational level. Regulation of gene expression at the translational level occurs for the yeast GCN4 (Hinnenbusch, 1984; Thireos et al, 1984), and the human thymidylate synthase (Sumiko et al, 1987) genes. However, the location of the control sequences implicated in the regulation of these two genes is found within the 5' leader sequence of the mRNA. The location of a positive element within the open reading frame of the TMP1 gene, which acts translationally or post-translationally, is a novel observation.

If the TMP1 DAS acts translationally, enhancement of TMP1 expression could be mediated by a factor affecting translational elongation. This putative trans-acting factor could interact with thymine nucleotides, e.g. dTMP or dTTP

or both, as cofactors and be allosterically modified to affect translation of mRNA. This could be another level of regulation to ensure the appropriate levels of thymine nucleotides. Support for this hypothesis is suggested by E. Calmels findings (M.Sc., 1987) which showed that relative to the untransformed strain AH22, a pTL1 transformant produced about 40 times more mRNA but only 10 times more thymidylate synthase activity. This hypothesis could be tested by constructing a pRS759-like integrant (pRS2107) and transforming this strain with the TMP1 gene on a high copy number plasmid. Reduction of β-galactosidase expression, without reduction of mRNA levels, when the strain harbours many copies of the TMP1 gene would suggest that the translational activator is titrated out by the additional copies of the TMP1 gene.

Periodic expression of TMP1 is independent of genomic location and an ARS element

To confirm that the sequences necessary for periodic expression are found within the 270 bp 5' flanking region, and not due to some fortuitous juxtaposition of sequences, the 2 micron plasmid sequence that conferred autonomous replication in yeast was removed (Russell et al, 1986). The resulting integrating plasmids were then targeted to either the TMP1 or the leu2 chromosomal locus (figures 8 and 9). The site of plasmid integration and the number of plasmid copies were confirmed by Southern analysis (figures 10, 11 and 12). A comparison of the β-galactosidase activity

expressed from the leu2 integrants with the TMP1 integrants suggested that the integrants with the TMP1'-'lacZ gene fusions at the leu2 locus expressed β-galactosidase at lower levels (table 4). Although expression was lowered, the β-galactosidase was expressed periodically during a synchronized cell cycle (M. T. Greenwood, M.Sc., 1986; R. W. Ord, Ph.D., 1987). Therefore, the information specifying cell cycle dependent expression is located within the -270 bps flanking the TMP1 open reading frame, and is not dependent on genomic location. This is consistent with Hereford and Osley's finding which showed that a H2A'-'lacZ gene fusion, when integrated at either the TRT1 or leu2 locus, was transcribed periodically (Osley et al, 1986). They also found that the level of β-galactosidase from the gene fusion at the leu2 locus was at least four-fold lower than that found when the gene fusion was present at the TRT1 locus (Osley et al, 1982).

Conflicting results concerning the role of the region between positions -376 bp and -270 bp

Conflicting results were obtained for the role of the region between -376 bp and -270 bps. First, the analysis of integrants harbouring disrupted chromosomal TMP1 genes suggests that this region is important for TMP1 expression (table 4; P. Poon, pers. comm). That is, when the levels of thymidylate synthase activity expressed by the integrants are compared with that expressed by the untransformed strain AH22, it was found that disruption of the TMP1 gene at

position -376 (RS561) did not affect expression while disruption at position -270 (RS456) reduced expression at least 4-fold (figure 8, table 4). This result suggests that the region between the positions -376 and -270 is important for TMP1 expression.

On the other hand, both the plasmids pTL30 and pRS535 expressed β-galactosidase at the same level although pRS535 contains this information and pTL30 does not (figure 6, table 3). This result suggests that the region between -376 and -270 is not important.

This apparent discrepancy may be due to context effects produced by having different sequences adjacent the TMP1 gene in the integrants RS561 and RS456. Also, the sequences adjacent to the position -270 in the autonomously replicating plasmids, pTL30 and pRS535, are different which could affect TMP1'-'lacZ gene expression (R. Ord, Ph.D., 1987). One approach to resolve this controversy is to construct another TMP1 integrant which has the same sequences next to the TMP1 gene as in RS561 but is missing the region between positions -376 and -270 bp. If thymidylate synthase activity expressed by this strain is lower than the activity levels expressed by RS561 or AH22, this would indicate that this region is important for TMP1 expression.

Two mechanisms are involved in inactivating thymidylate synthase activity

The results presented here suggest there are two distinct mechanisms involved in inactivating thymidylate synthase. During the early to midlog stages of batch culture growth, there is an energy independent inactivation of the hybrid thymidylate synthase - β-galactosidase enzymes, while in the later stages of batch growth inactivation is energy dependent (tables 8 and 9).

M. T. Greenwood found that as yeast cultures entered stationary phase of batch culture growth, thymidylate synthase decayed at a faster rate than did the bulk cellular proteins. Also, he found that hybrid thymidylate synthase-β-galactosidase proteins containing more than 46 amino acids of thymidylate synthase N-terminal region were inactivated by a mechanism that required energy (M.Sc., 1986; Greenwood et al, 1986). Therefore, he proposed that thymidylate synthase may be inactivated by the ATP-requiring ubiquitin proteolytic pathway (Ciechanover et al, 1984).

If this is the case, it seems unusual that there would also be an energy independent inactivation operating during the early to midlog stages of batch culture growth (tables 8, 9 and 10). This mechanism appears to predominate during the early phases of logarithmic growth. One possible reason is that this type of proteolysis may be specific for the inactivation of those proteins whose levels must fluctuate during the cell cycle. In addition, the polypeptide region

between the 46th and 112th amino acids of thymidylate synthase appears to contain the signal(s) for inactivation by both the energy independent and dependent mechanisms (table 7). A decision as to whether there is one or two distinct regions which specify instability awaits further analysis.

Cell cycle regulation

The TMP1 message is periodically expressed during the cell cycle (Storms et al, 1984). Since the periodic regulation of gene expression occurs by controlling transcription (Osley and Lycan, 1987; Nasmyth et al, 1987a; Nasmyth et al, 1987b), at least one of the factors interacting at the promoter region could modulate TMP1 transcription in a cell cycle dependent fashion. The putative repressor and/or activator mentioned earlier are possible candidates for this role.

Interestingly, transcription of TMP1 occurred during the refeeding of stationary phase cells in the presence of the protein synthesis inhibitor, cycloheximide (Calmels, 1987). One interpretation is that the positive factor is expressed constitutively while the negative factor is synthesized periodically. A possible reason is that a basal level of thymidylate synthase is needed to synthesize dTMP for other cellular processes such as DNA repair. Furthermore, the MluI fragment has two redundant sequences 5'-TGACGCGTT-3' which are also found in the upstream regions of two other cell cycle regulated genes: CDC9 which encodes DNA ligase

(Peterson et al, 1985) and CDC8 which encodes for thymidylate kinase (White et al, 1987). Since these three genes are expressed coordinately during the cell cycle (White et al, 1987), this sequence may be important for signaling a common factor involved in their coordinate control.

The TMP1 mRNA decreases to nondetectable levels during stationary phase. Other critical trans-acting factors such as a TATA binding factor or initiation factors could be the components that disappear during stationary phase and consequently, no TMP1 mRNA is detected despite the presence of the positive factor. An alternative explanation is that one, or more than one, component of the transcription machinery is reversibly inactivated during stationary phase. This would suggest that cells reactivate the transcription machinery following refeeding.

Further Experiments

The results presented here suggest at least two trans-acting factors would bind DNA in the 270 bp sequence upstream of the TMP1 open reading frame. One factor has a positive role and recognizes DNA sequences within or overlapping the region between -159 and -122 bp, while the other factor has a negative role and interacts in the region between -270 and -159 bp. These two regions were required for specifying normal TMP1'-'lacZ expression (table 3). Since these factors interact in the region upstream of the open reading frame, their role in regulating TMP1 expression

is most likely at level of transcription.

Since the regulatory regions have not been identified precisely, further experiments should be performed to define their DNA recognition sequences. These regions should be localized more precisely by deletion analysis, linker scanning mutagenesis, and *in vitro* mutagenesis. Deletion analysis and linker scanning mutagenesis would define the boundaries of the regulatory regions, while *in vitro* mutagenesis would define the critical nucleotides within the regions that regulate *TMP1* expression. The upstream regions defined by this analysis are potentially the DNA binding sites used by the *trans*-acting transcription factors to regulate *TMP1* expression.

Two biochemical approaches can be used to verify whether the DNA sequences identified are sites where proteins interact with the *TMP1* gene. One approach would utilize the gel retardation assay. Although this approach does not identify the exact sequences which are important for the binding of *trans*-acting factors, it is useful for determining whether the DNA fragment has sequences which are bound by DNA sequence specific binding proteins. Once gel retardation has established that proteins interact with these regions, methylation interference experiments can be used to define the sequences which interact directly with proteins. A methylation protection study would determine the G and A residues which make contact with the DNA sequence specific protein.

The localized regulatory regions can be used as "substrates" for the purification of *trans*-acting factors using DNA-affinity columns. By covalently linking the DNA fragment to the matrix of an affinity column, the DNA sequence specific protein in a crude extract which binds the DNA fragment will be selectively purified. Once purified, the protein can be used to produce either polyclonal or monoclonal antibodies. These antibodies can then be used in conjunction with an *E. coli* expression library of yeast genomic DNA to clone the genes encoding for the DNA binding factors.

I feel these are the next important experiments for understanding the regulation of the *TMP1* gene.

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