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Factors Effecting the Levels of Thymidylate Synthase in
Saccharomyces cerevisiae

Michael T. Greenwood

A Thesis
in
The Department
of
Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

April 1986

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ABSTRACT

Factors Effecting the Levels of Thymidylate Synthase in Saccharomyces cerevisiae

Michael T. Greenwood

Three different experimental approaches were used to study the mechanisms regulating thymidylate synthase levels in yeast.

(1) Direct measurement of thymidylate synthase levels during the different growth stages of batch cultures revealed that post-translational inactivation played a major role in regulating the levels of thymidylate synthase.

(2) The study of thymidylate synthase β -galactosidase fusion proteins localized an amino acid sequence on thymidylate synthase. This sequence apparently targeted thymidylate synthase for post-translational inactivation.

(3) A study of the effects of increases in the TMPl gene dosage showed that yeast cells have no apparent regulatory mechanism to compensate for an increase in the TMPl gene copy number. A variation from 0.5 to 15 times the normal steady state levels of thymidylate synthase was not detrimental to the cell.

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INTRODUCTION

Thymidylate metabolism

Of the 5 nitrogenous bases involved in nucleic acid metabolism only thymine is exclusively found in DNA (reviewed in Kornberg 1980). Perturbations in the cellular levels of thymidylate (dTMP) have been found to cause a great deal of damage. Starvation for thymidylate has been shown to lead to genetic damage and to cell death (thymineless death) in a wide range of organisms including E. coli (Cohen and Barner 1954), cultured fibroblasts (Koyama et al. 1982) and yeast (Barclay and Little 1978). In addition, increases in thymidylate pools have been shown to be mutagenic (Barclay and Little 1981) and lead to cell death (Tooper et al. 1981).

Regulation of thymidylate synthase levels

Thymidylate is made by the enzyme thymidylate synthase (E.C.2.1.1.45) which catalyzes the methylation of dUMP to form dTMP (Kornberg 1980). This reaction is the sole de novo source of thymidylate. The dramatic effects resulting from thymidylate pool imbalances and the periodic demand for thymidylate during DNA synthesis suggest that the levels of dTMP must be precisely regulated. It is therefore not surprising that the levels of enzymes involved in thymidylate production, including thymidylate synthase, have been shown to be highly regulated (reviewed

in Prescott 1976). In yeast, thymidylate synthase is a key enzyme, because unlike most other organisms, yeast does not have a salvage pathway for the synthesis of thymidylate (Griveil and Jackson 1968; Bisson and Thorner 1977).

As far back as 1960, Maley and Maley showed that thymidylate synthase levels were low in rat livers, but activity increased dramatically when liver cells were induced to divide in response to a partial hepatectomy (Maley and Maley 1960). The same phenomena was subsequently observed in many different cell types (for reviews see Kornberg 1981; Prescott 1976). In addition, thymidylate synthase levels were found to be elevated in rapidly growing cells such as tumor (Jackson and Niethammer 1979) and embryonic tissues (Parisi and DePetrocellis 1976).

More recently, Johnson's group have been studying the mechanisms involved in regulating thymidylate synthase levels in mouse 3T6 fibroblasts. When quiescent (G0) mouse 3T6 cells were stimulated with serum to divide, the low basal level of thymidylate synthase increased dramatically (Navalgund et al. 1980). It was further shown that elevated levels of thymidylate synthase were confined to the DNA synthetic phase of the cell cycle. Cell-cycle-stage dependent accumulation of thymidylate synthase activity has been observed in many other cell types including algae (Bachmann et al. 1983) and yeast (Storms et al. 1984). In mouse 3T6 cells the cell-cycle-stage dependent increase in

thymidylate synthase was probably due to new synthesis, because it was blocked by inhibitors of protein and RNA synthesis (Navalgund et al. 1980) and because an increase in thymidylate synthase activity was shown to be coupled to an increase in detectable thymidylate synthase specific mRNA (Jenhet et al. 1985). The low levels of thymidylate synthase in quiescent mouse 3T6 cells has been attributed to simple dilution caused by a fast decrease in the rate of thymidylate synthase synthesis while total protein synthesis continued at a high rate. Direct measurements on the stability of thymidylate synthase in mouse 3T6 cells demonstrated that the protein is more stable than the bulk of cellular proteins (Navalgund et al. 1980; Jenhet et al. 1985).

The results presented by our own lab (Storms et al. 1984) suggested that the regulation of thymidylate synthase synthesis in yeast cells was similar to what is observed in mouse 3T6 cells. Thymidylate synthase levels were found to be elevated during S phase. Since a periodic increase in thymidylate synthase specific mRNA preceded the increase in thymidylate synthase activity, it was argued that the cell-cycle dependent accumulation of thymidylate synthase was due to new synthesis. In contrast to the results obtained with mouse 3T6 cells, inactivation seemed to play a role in governing cellular levels of thymidylate synthase in yeast. This was concluded after observing a loss of

4.
thymidylate synthase activity, measured per ml of synchronous culture, after S period.

In a human gastrointestinal cell line, thymidylate synthase activity was seen to decay with a half life of about 6 hours following the inhibition of protein synthesis with cycloheximide (Washtien 1984). The observed decay rate of thymidylate synthase, following the addition of cycloheximide, in the human cell line is about four times faster than the observed decay rate in mouse 3T6 cells (half life of about 25 hours; Jenh et al. 1985; Navalgund et al. 1980). These conflicting results suggest that the in vivo stability of thymidylate synthase may be cell type specific.

Post-translational regulatory mechanisms

Elaborate transcriptional and translational mechanisms exist to control the amount of enzyme synthesis. Once an enzyme is synthesized, post-translational events are involved in controlling the level of enzymatic activity. Allosteric inhibition, covalent modification (i.e. phosphorylation), and protein degradation are known to be involved in modulating enzymatic activities. The irreversible nature of protein degradation is useful in differentiating it from the reversible protein modifications. To illustrate this point we can look at the inactivation of fructose-1,6-bisphosphatase in yeast. It

was observed that the levels of this gluconeogenic enzyme were high in cells growing on ethanol but the levels were low in cells growing on glucose. The addition of glucose to a yeast culture growing on ethanol resulted in a 50 to 70 % decrease in activity within 3 minutes followed by a much slower disappearance of the remaining activity (Lenz and Holzer 1980). The initial drop in activity was due to phosphorylation and full levels were quickly restored, even in the presence of cycloheximide, if the glucose was quickly removed. In contrast, activity is irreversibly lost 30 minutes after the addition of glucose due to the degradation of the enzyme (Purwin et al. 1982; Mazon et al. 1982). This was concluded because loss of activity paralleled the loss of fructose-1,6-bisphosphatase antigen.

Protein degradation is believed to occur via lysosomal and non-lysosomal pathways (reviewed in Ciechanover et al. 1984). The lysosome or vacuole is an organelle containing a large number of different catabolic enzymes including RNases, lipases, and proteases. Biochemical and genetic evidence have shown that the vacuole is responsible for general protein degradation such as the turnover of long lived proteins. Vacuolar based proteolysis has also been shown to play a major role under conditions of nutritional deprivation. The non-lysosomal pathway is believed to be responsible for selective degradation of short lived and aberrant proteins. Ubiquitin

is a 76 amino acid polypeptide which has been highly conserved during evolution (Ozkaynak et al. 1984). The covalent attachment of ubiquitin has been shown to target proteins for non-lysosomal degradation (reviewed in Ciechanover et al. 1984). It is not yet known if all non-lysosomal protein degradation occurs via the ubiquitin pathway. Furthermore, ubiquitin mediated protein degradation has been shown to occur in only a few cell types (Ciechanover et al. 1984).

Most proteins are subjected to intracellular degradation (reviewed in Goldberg and St John 1976). In yeast, evidence has been presented which demonstrated that as much as 80% of the total protein is subject to degradation (Lopez and Gancedo 1979). The degradation rate of individual proteins reflects the metabolic importance of the enzyme. Enzymes catalyzing the first or the rate determining step of a metabolic pathway usually have short half lives (reviewed in Goldberg and St John 1976). In addition, the rate of general protein degradation and the rate of degradation of individual proteins has been shown to be greatly effected by growth conditions. Yeast cells growing on glucose showed a basal rate of protein degradation of between 0.5 to 1% per hour. Upon starvation, the basal rate increased 2 to 3 fold (Lopez and Gancedo 1979). Most, if not all, the increase was due to an increase in vacuolar based proteolysis (reviewed in Jones

1983). It is not known if the increase is due to to the enhanced degradation of all proteins or a subset of yeast proteins.

Protein degradation has been shown to have an absolute requirement for cellular energy (reviewed in Goldberg and St John 1976). The energy requirement probably reflects the selectivity of the non-lysosomal pathway, and the necessity to maintain a low pH within the lysosome. A low pH is required because the vacuolar based proteases are largely inactive at neutral pH.

Experimental design

Although a considerable amount of descriptive work has been done, very little is known about the regulatory events controlling levels of thymidylate synthase. In order to rectify this situation, three separate experimental approaches were used to gain insight into the mechanisms governing thymidylate synthase levels in yeast.

The first approach involved monitoring enzyme activity during batch culture growth and when cell cycle progression is arrested in G1 with the yeast pheromone α -factor. This analysis showed that thymidylate synthase levels are elevated in rapidly proliferating cells and reduced in quiescent or G1 arrested cells. Inactivation of existing activity played a major role in governing thymidylate synthase. In addition, preliminary evidence is

presented which suggests inactivation, occurred by a selective mechanism not affecting the bulk of cellular proteins.

The second approach involved the use of TMP1 - LacZ gene fusions. The hybrids consisted of a constant portion of the E. coli LacZ structural gene fused to increasingly larger portions of the yeast TMP1 gene. The fusion genes encode hybrid proteins consisting of 13, 97, 110, or 190 N-terminal amino acids of thymidylate synthase fused to the last 1015 C-terminal amino acids of B-galactosidase. All the fusion proteins are expressed from identical TMP1 promoters. Such a genetic approach has proven useful in identifying the regulatory sequences involved in the transcriptional control of a variety of yeast genes (Guarente 1983; Rose and Bostein 1983; Casadaban et al. 1983). Similarly, the TMP1 - LacZ fusion genes have allowed the identification of a regulatory sequence responsible for the periodic accumulation of thymidylate synthase (Ord, R.W., PhD thesis, Concordia University, in preparation). The availability of hybrid genes encoding hybrid proteins has also allowed the identification of amino acid sequences responsible for such functions as nuclear targeting (Hall et al. 1984; Silver et al. 1984; Gritz et al. 1985; Moreland et al. 1985), mitochondrial targeting (Douglas et al. 1984), and DNA binding (Johnson and Herskowitz 1985). Here a series of

TMP1 - LacZ hybrid genes was used to identify an amino acid sequence involved in the post-translational inactivation of thymidylate synthase activity.

Finally, the cloned TMP1 gene was used to determine the effect of increasing the TMP1 gene dosage on levels of thymidylate synthase and on the physiology of the cell. Such an approach has proven useful in identifying post-transcriptional and post-translational regulatory mechanisms involved in regulating levels of ribosomal proteins (Abovich et al. 1985; Warner et al. 1985) and of histones (Osley and Hereford 1981).

MATERIALS AND METHODS

Strains

The strains used in this study are shown in table 1. Many of the strains listed in table 1, were also used when they were transformed with different plasmids. Throughout this text, I will use the following nomenclature for the transformants. The name of the strain will be followed by the name of the plasmid. Thus strain AH22-pTL1 will refer to strain AH22 transformed with plasmid pTL1.

The diploid strains RS421 and RS422 were constructed as follows. The haploid cells to be mated were mixed together and incubated for 48 hours at 30°C on a YEPD nutrient plate (rich media, see below) to allow mating. Diploids were selected by streaking on minimal media plates (YNBD, see below) supplemented with leucine (10 µg/ml). The diploid strain obtained by mating BB30 (leu2⁻, tmpl⁻) with LL20 (leu2⁻, his3⁻), RS421, was selected for growth without exogenously supplied histidine and deoxythymidine 5' monophosphate (dTMP) and then screened for its inability to grow without leucine. The diploid strain obtained by mating AH22 (leu2⁻, his4⁻) with LL20 (leu2⁻, his3⁻), RS422, was selected for growth without exogenously supplied histidine and then screened for its inability to

Table 1

Saccharomyces cerevisiae strains used.

Strain	Genotype	Source
S288c	α <u>SUC2</u> <u>mal</u> <u>mel</u> <u>gal2</u> <u>CUP1</u>	Yeast Genetic Stock
LL20	α <u>leu2-3</u> <u>leu2-112</u> <u>his3-11</u> <u>his3-15</u>	G.R. Fink
2180	α <u>gal2</u> <u>trp1</u> <u>pep4-3</u>	Yeast Genetic Stock Center
4A	<u>a</u> <u>leu2-3</u> <u>leu2-112</u> <u>pep4-3</u> <u>his4-419</u> <u>trp1</u>	Sym Davis- Haploid cell obtained by sporulating a diploid (20B-12 x AH22).
BB30-3	<u>a</u> <u>leu2-3</u> <u>leu2-112</u> <u>trp1</u> <u>tup+</u>	J.G. Little
RS421	<u>a</u> / α <u>trp1/+</u> <u>tup+/-</u> <u>leu2/leu2</u> <u>+ /his3</u>	This study- Diploid cell obtained by crossing LL20 with BB30-3.
RS422	<u>a</u> / α <u>can1/+</u> <u>leu2/leu2</u> <u>+ /his3</u> <u>his4/+</u>	This study- Diploid cell obtained by crossing AH22 with LL20.
AH22	<u>a</u> <u>can1-1</u> <u>leu2-3</u> <u>leu2-112</u> <u>his4-514</u>	G.R. Fink

grow without leucine. The complete genotypes of the diploid strains are shown in table 1. The strains RS421 and RS422 were shown to be diploid cells by their ability to sporulate in a nitrogen poor growth media (Sherman et al. 1979).

Plasmid construction

The plasmids used in this study are shown in table 2. Here, I will describe the nature and construction of plasmids pRS264i and pTL1'. The construction and characterization of the other plasmids will be described elsewhere (see table 2).

Plasmid pTL1 contains a 10 Kb Hind III to Hind III insert of yeast chromosomal DNA containing the TMP1 gene (Taylor et al. 1982). The insert is cloned within a unique Hind III site of a yeast and E. coli shuttle vector. In plasmid pTL1, the 5' end of the TMP1 gene lies adjacent to the yeast 2 μ origin of replication. In plasmid pTL1', the 10 Kb Hind III to Hind III fragment containing the TMP1 gene is inverted with respect to the vector sequence so that the 5' end of the TMP1 gene now lies adjacent to the LEU2 gene. To construct plasmid pTL1', pTL1 plasmid DNA was digested with Hind III, ligated and transformed into E. coli. Plasmid DNA from the transformants was isolated, and pTL1' was identified by endonuclease analysis.

Plasmid pRS264 contains a TMP1 - lacZ fusion

Table 2
Plasmids used

Plasmid	Pertinent features	Source
PRS535	pBR322 <u>AMPr</u> <u>2μ</u> <u>LEU2</u> <u>TMP1-lacZYA</u> (15.9 Kb)	R.W. Ord
PRS757	same (16.1 Kb)	Yip Ho
PRS269	same (16.2 Kb)	R.W. Ord
PRS264i	same (16.4 Kb)	This study
pYT760-ryp3	pBR322 <u>AMPr</u> <u>2μ</u> <u>LEU2</u> <u>ryp3-lacZYA</u> (15.6 Kb)	D. Thomas
pTL1	pBR322 <u>AMPr</u> <u>2μ</u> <u>LEU2</u> <u>TMP1</u> (23.4 Kb)	Taylor et al. 1982
pTL1'	same (23.4 Kb)	This study
PRS(cen3)471	pBR322 <u>ars1</u> <u>cen3</u> <u>LEU2</u> <u>TMP1</u> (10.2 Kb)	E.M. Calmels

gene. The fusion consists of the lacZ structural gene under the transcriptional and translational control of the TMP1 gene. DNA sequences of both the lacZ and TMP1 genes were used to determine that the fusion junction is not in the correct translational reading frame (R. W. Ord, PhD thesis, Concordia University, in preparation). To construct plasmid pRS264i, pRS264 plasmid DNA was digested with Bam HI, which cut the plasmid only at the TMP1 LacZ junction. The 4 base pair single stranded Bam HI generated sticky ends were removed by S1 nuclease digestion and the subsequent blunt-ended DNA was ligated. Plasmid pRS264i was therefore identical to plasmid pRS264 except that the junction on the TMP1 - lacZ fusion gene was in the correct translational reading frame. All manipulations involving DNA were as described in Maniatis et al. 1982.

Growth medias

2x YNBD consists of 1.34% Yeast Nitrogen Base (without amino acids, Difco), 4% glucose, and 10 µg/ml of the required amino acids (Osley and Hereford 1981). YEPD consists of 2% bactopectone, 1% yeast extract, and 4% glucose. To obtain aerobic growth, yeast cells were grown in YEPG (Sprague and Cronan 1977; Dujon 1981). YEPG is identical to YEPD except that 4% glycerol replaces the 4% glucose. 0.1% consists of 0.1% bactopectone, 0.1% yeast extract, 1.34% Yeast Nitrogen Base (without amino acids),

4% glucose and 10 µg/ml of the required amino acids (Storms et al. 1984).

Growth of yeast strains

All growth and culture manipulations (i.e. refeeding and α -factor arrest experiments) were accomplished in a 30°C "walk in" type incubator room. This allowed the elimination of perturbations due to temperature shock. Maximal aeration was maintained with a high flask volume to culture volume ratio (5:1). For routine growth, cells were grown on the minimal media called 2x YNBD. Cells from a saturated 2 ml overnight culture (1×10^8 cells/ml) were used to inoculate 25 to 200 ml of media at approximately 1×10^5 cells/ml. After overnight growth for a minimum of 5 generations, the cultures were considered to be growing exponentially as long as the OD 600 of the culture remained below 0.4. Growth of the cultures was followed by measuring the optical density, using a 1 cm cuvette, at 600nm on a LKB ultrospec 4050 or by following increases in turbidity with a Klett colorimeter. 1 to 12 ml culture samples were harvested by centrifugation (3 minutes in an IEC Clinical Centrifuge), frozen in liquid nitrogen and stored at -80°C. These samples were then used as a source of material for determining total protein content, thymidylate synthase activity, and β -galactosidase

activity. All strains were maintained on 2x YNBD media plates (2% agar) with monthly restreaking to confirm phenotypes. The plated strains were used to set up culture overnights (see above).

Stationary phase cultures that had lost upwards of 90% of their thymidylate synthase activity were used as source cells in the refeeding experiments. Stationary phase cells were resuspended in fresh prewarmed 2x YNBD media with and without 100 µg/ml of cycloheximide. At various times thereafter samples were harvested and stored as described above.

α-factor arrest

α-factor arrest was as described by Hereford et al. (1981). The supernatant of the saturated culture of an mating type strain was used as the source of α-factor. Strain S288C was inoculated in 200 ml of YNBD media (0.67% YNB w/o amino acids and 2% glucose) with cells taken directly from a freshly streaked plate (YNBD plus 2% agar). The initial cell density was about 1×10^6 cells/ml. After the cultures were grown with vigorous shaking on a reciprocal shaker at 30°C for 48 hours, the cells were removed by centrifugation and the supernatant filter-sterilized (Nalgene Filtunit, 0.45 µm). An equal volume of growth media was added (0.2% yeast extract, 0.2% bactopectone, 8% glucose, and 20 µg/ml of the required amino acids) to the sterilized culture

supernatant. This mixture is the " α -factor arresting media" and was made fresh for each experiment. Therefore exponentially growing cultures of the strains to be arrested were required at the time the α -factor was ready. To accomplish this, a 2 ml culture of the required strain was grown, in 2x YNBD media, to be used as a source of inoculum. The following day, the 2 ml culture was saturated (1×10^8 cells/ml) and it was used to inoculate 200 to 400 ml of 0.1% media at around 5×10^2 cells/ml. The cultures were allowed to grow to a cell density of between 3 and 5×10^6 cells/ml. 200 ml of the exponentially growing culture was spun down (30°C , 5 minutes) and resuspended in 400 ml of " α -factor arresting media". The cultures were then aerated at 30°C on a rotary shaker and cell samples were harvested at $t=0$ and at subsequent time intervals until G1 release.

Bud morphology

Bud morphology was used to judge the degree of G1 arrest because of the distinct coupling of bud size and cell cycle stage (Williamson and Scopes 1961). This was accomplished by the microscopic examination of formaldehyde fixed cell samples. The cell samples were sonicated to dissociate clumped cells. At least 300 cells were examined for each time point.

Thymidylate synthase assays

Thymidylate synthase activity was determined using 3 methods, one used permeabilized cells and the others used cell extracts.

a) Permeabilized cells . Thymidylate synthase activity levels per ml of culture were determined by the radiochemical procedure measuring the release of tritium from [5-³H]-dUMP during the formation of dTMP, following the modifications of Bisson and Thorner (1977). For this, frozen cell samples were resuspended in 100 μ l of cold buffer A (20% glycerol, 10mM 2-mercaptoethanol, 10mM MgCl₂, 1mM EDTA, and 20mM Tris-HCl pH7.4) containing 3% Brij 35 to permeabilize the cells. The samples were then placed at 25^oC and 100 μ l of prewarmed buffer B (200 μ M [5-³H]-dUMP around 2×10^4 dpm/nmol, 1.4mM THFA, 40mM formaldehyde, 20mM MgCl₂, 2mM EDTA, 0.2M 2-mercaptoethanol, and 0.2M Tris-HCl pH 7.4) was added to initiate the reaction. After 30 minutes of incubation, the reaction was terminated by the addition of 500 μ l of 12% charcoal in 0.1 N HCl. After 30 minutes of incubation on ice, the samples were filtered through nitrocellulose disks (Millipore, 0.45 μ m). The levels of tritium labelled water (³H₂O) produced during the assay was quantitated by scintillation counting a portion of the filtrate (Bray 1960). One μ Unit of activity is defined as 1 pmole of ³H released per minute (Storms et al. 1984). The assay was found to be linear with time (up to 35 minutes) and with the amount of

cell material (up to 10^9 cells) (data not shown but similar to Bisson and Thorner 1977). The release of $^3\text{H}_2\text{O}$ from $[5\text{-}^3\text{H}]\text{-dUMP}$ was completely inhibited by the stoichiometric thymidylate synthase inhibitor FdUMP. All thymidylate synthase values shown are the averages of at least two independent samples.

b) Cell extracts . Crude extracts were obtained by resuspending frozen cell pellets in 2 to 3 volumes of buffer A followed by vigorous vortexing with acid washed glass beads (300 to 600 μM , John's Scientific) at 4°C for 5, one minute intervals. Cellular debris and unbroken cells were removed by centrifugation (4°C , 16K, and 30 minutes). The supernatant was used as the source of crude cell extracts. The protein content of the extracts was determined using the Lowry assay (Lowry et al. 1951). The extracts could be stored at -80°C for several weeks without loss of thymidylate synthase activity. The extracts were used to quantitate thymidylate synthase activity by two independent methods: (1) by the levels of ^3H released during assay with $[5\text{-}^3\text{H}]\text{-dUMP}$ and (2) by the levels of $[6\text{-}^3\text{H}]\text{-FdUMP}$ binding. The ^3H release assay using crude extracts was essentially as described above. The FdUMP binding assay is essentially as described by Belfort et al. (1983). The assay is based on the formation of a stable ternary complex involving $[6\text{-}^3\text{H}]\text{-FdUMP}$, N5N10-methylenetetrahydrofolate and

[6-³H]-FdUMP, N5N10-methylenetetrahydrofolate and thymidylate synthase. Since FdUMP binds thymidylate synthase in a stoichiometric fashion, the levels of thymidylate synthase can be determined by polyacrylamide gel electrophoresis of [6-³H]-FdUMP treated total soluble protein and subsequent fluorography. The [6-³H]-FdUMP binding assay was essentially as described for the ³H release assay except that [6-³H]-FdUMP replaced the [5-³H]-dUMP. SDS polyacrylamide electrophoresis of [6-³H]-FdUMP treated crude yeast cell extracts was accomplished by Reno Clarizio. The detailed protocols can be found elsewhere (R. Clarizio, M.Sc. thesis, Concordia University, in preparation).

Total protein

Total protein content per ml of culture were determined using a modification of the Lowry protein assay previously described (Steward 1975). Frozen cell pellets were washed and resuspended in 1ml of cold distilled deionized water. For each time point, 3 independent samples consisting of 25, 50, and 100 μ l of the cell suspension were assayed. 500 μ l of 1M KOH were then added and the final volumes brought up to 1 ml with distilled deionized water. After vortexing, the mixtures were placed in a boiling water bath for 11 minutes (this was determined to be the optimal time for strain AH22, data not shown). After the tubes cooled to room temperature, 2

ml of a freshly made copper solution (50ml of 2% NaCo₃ and 4ml of 0.125% CuSO₄:5H₂O in 0.25% KNa tartrate) was added and the samples were incubated at room temperature for 10 minutes when 0.5 ml of phenol reagent (Folin-Ciocalteu phenol reagent, Fisher) was vigorously added and immediately vortexed. It should be noted that water is added to the commercially available phenol reagent to make it 1.0 M with respect to acid. This is determined by titration with KOH to a phenolphthalein end point (i.e. 0.31 mls of H₂O and 0.19 mls Folin-Ciocalteu phenol reagent, for each reaction). The samples were then incubated at room temperature for at least 30 minutes. At the end of the incubation period the samples were centrifuged for 3 minutes in an IEC Clinical Centrifuge (room temperature) to remove the white inorganic precipitate that forms. Absorbances of the final solution was determined at 540 nm (LKB Ultrospec 4050) and protein content per ml of culture was determined using a standard curve constructed by assaying 25 to 150 µg of bovine serum albumin. The assay was linear up to a total of about 1×10^7 haploid AH22 cells.

B-galactosidase assays

B-galactosidase was assayed essentially as described by Miller (1972). Frozen cell samples were resuspended in 1 ml of Z buffer (0.06M Na₂HPO₄, 0.04M

NaH₂PO₄, 0.01M KCl, 0.001M MgSO₄, 0.05M β-mercaptoethanol, and HCl to adjust the pH to 7.0) containing 0.8 mg/ml of ONPG (Sigma Biochemicals) and 3% Brij 35 (Fisher) to permeabilize the cells. When a faint yellow color was detectable, the assay was terminated by the addition of 0.5 ml of 1M Na₂CO₃. The cells were then removed by centrifugation for 5 minutes in an IEC Clinical Centrifuge, and the OD at 420 nm measured. 1 unit of β-galactosidase activity is defined as 1 pmole of ONPG cleaved per minute. All β-galactosidase activity values shown are the average result obtained from assaying at least 2 independent samples. It should be noted that for the purpose of this assay, cell samples required prior freezing at -80°C in order to obtain maximum levels of activity with Brij 35.

Yeast transformation

Yeast cells were transformed with plasmid DNA using the LiCl method (Ito et al. 1983). Log phase cells growing in YEPD media were harvested in late log phase (around 2×10^7 cells/ml), washed once in TE (1mM EDTA, 10mM Tris-HCL pH 7) and resuspended in TE at about 2×10^8 cells/ml. An equal volume of cells and of 0.2M LiCl were then mixed together and the mixture was incubated for 1 hour in a shaking 30°C water bath. Plasmid DNA (1-2 μg) was then added and the cells were incubated at 30°C for 30 minutes. The cell samples

were then diluted with an equal volume of 70% polyethylene glycol-4000 and incubated without shaking at 30°C for 1 hour. After 5 minutes at 42°C, the samples were washed twice with distilled deionized water and the cells were plated on selective media at about 10^6 cells/plate. Because the plasmids used in this study contain the yeast LEU2 gene and all the strains transformed have a double mutation in the LEU2 gene, all transformants were selected for on media without leucine. Furthermore because of the double mutation in the LEU2 gene the reversion rate is extremely low (less than 10^{-10}). Since the rate of transformation is between 10^{-4} to 10^{-5} , the leu⁺ colonies obtained by DNA transformation are true transformants. Transformants usually appeared as distinct colonies after 3 to 5 days of incubation at 30°C on YNBD plates. These colonies were then restreaked on selective YNBD agar plates to purify the transformants.

RESULTS

SECTION 1: Factors effecting the levels of thymidylate synthase

In several eukaryotic systems, thymidylate synthase activity is elevated in rapidly dividing cells and low in quiescent cells. In this work, this variation in thymidylate synthase activity was demonstrated in yeast and the mechanisms by which variation was accomplished were investigated.

Thymidylate synthase levels during entry into stationary phase

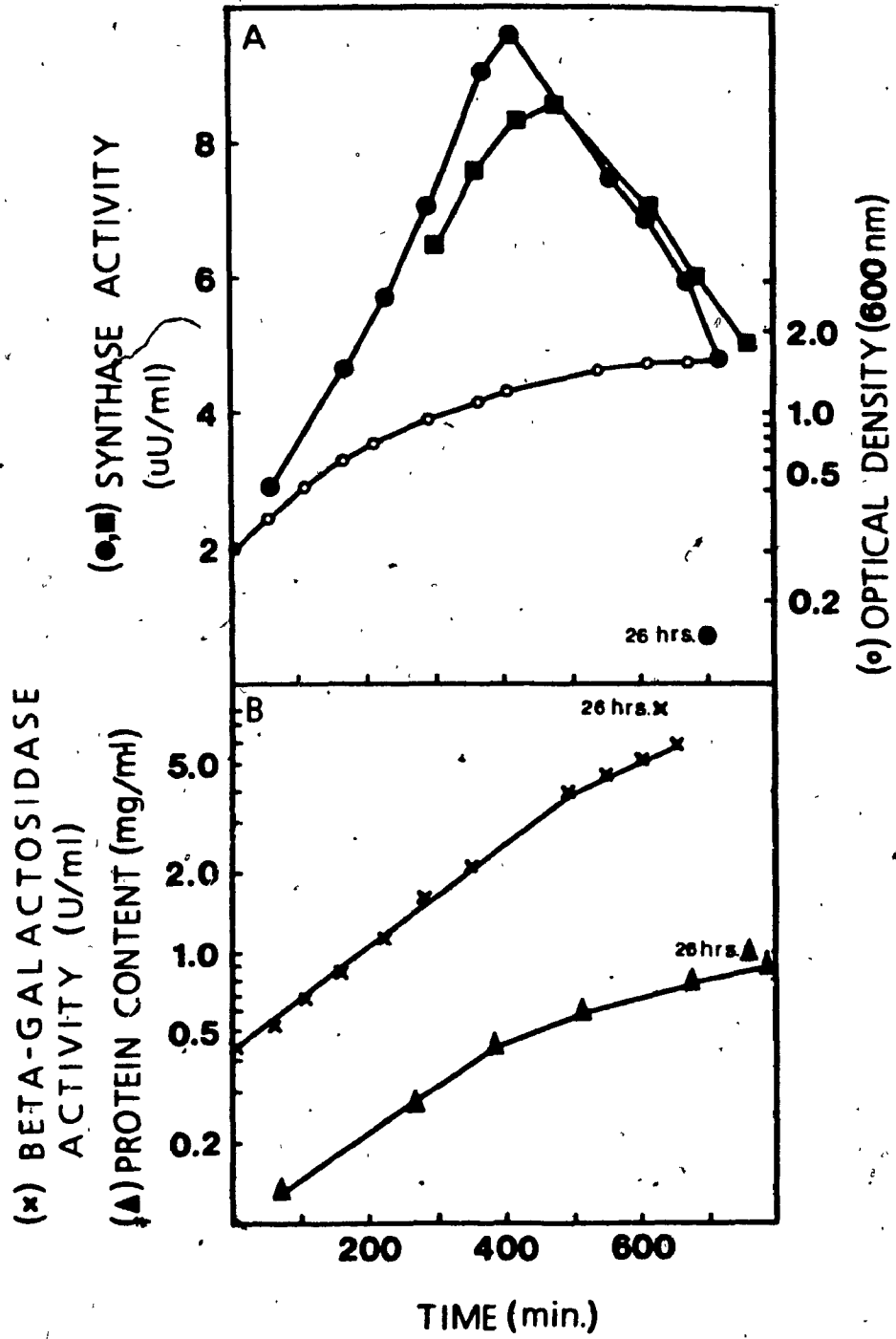
Thymidylate synthase levels, protein content, and culture turbidity (OD at 600nm) were monitored as an exponentially growing culture of strain AH22 entered stationary phase (fig. 1). Thymidylate synthase activity, per ml of culture, peaked when the culture reached an OD 600 of about 1.0. During the following 300 minute interval, 50% of the activity was lost although protein content and culture turbidity continued to increase (fig. 1). Further incubation resulted in the culture density reaching an OD 600 around 2.0 and over 90% of the synthase activity disappearing. In contrast to the thymidylate

FIGURE 1

Thymidylate synthase activity, total protein, and β -galactosidase activity during the transition from logarithmic growth to stationary phase. Cells were grown overnight in 2x YNBD media and samples were harvested at various intervals until stationary phase was reached. The final samples were harvested 26 hours after the initial ($t=0$) samples.

A: growth curve as determined by measuring the turbidity of the culture with a spectrophotometer at O.D. 600nm and thymidylate synthase activity levels per ml of culture in AH22 (circles) and in 20B-12 (squares) as determined in Brij 35 permeabilized cells using the radiochemical procedure. The growth curves for RS290 and 2180 were identical to the one shown for AH22. The results shown are similar to the results obtained when the experiments were repeated.

B: levels of total protein per ml of culture in AH22 as determined by the modified Lowry assay (Steward 1975), and β -galactosidase activity levels per ml of culture as assayed as described by Miller (1972) in strain AH22-pYT760-ryp3. Experiment was not repeated.



synthase activity profile obtained (fig. 1A) total protein increased throughout paralleling the growth curve (fig. 1B).

To further monitor protein synthesis during entry into stationary phase, levels of β -galactosidase were followed in strain AH22 transformed with plasmid pYT760-ryp3 (called strain AH22-pYT760-ryp3). The β -galactosidase activity profile obtained was very similar to the pattern observed for total protein (fig. 1B).

The results presented in fig. 1 have shown that thymidylate synthase levels decreased, when measured in Brij 35 permeabilized cell samples, during the entry into stationary phase. To show that the observed loss of thymidylate synthase activity is not due to differential permeabilization by Brij 35, the levels of thymidylate synthase were determined in cell free extracts. These results (table 3) confirmed that thymidylate synthase activity was lost as yeast cultures left logarithmic growth and entered stationary phase.

To determine if the loss of thymidylate synthase activity was a vacuolar based event, thymidylate synthase activity was monitored when a pep4-3 mutant entered stationary phase. Strains carrying the pep4-3 mutation have drastically reduced levels of several vacuolar based hydrolases including the major proteases (reviewed in Jones 1983). The pep4-3 mutant phenotype of strain 2180

Table 3

Thymidylate synthase specific activity during entry into stationary phase^a

	Source of extracts	thymidylate synthase activity (μ U/mg)
A	exponential phase	21.1
B	early stationary phase ^b	10.5
C	mid stationary phase ^c	6.8
D	late stationary phase ^d	0.1

^a Strain AH22-pYT760-ryp3 was grown overnight in 2x YNBD media as described in methods and materials. At various times during batch culture growth, samples were harvested and crude cell free extracts prepared as described in materials and methods. Protein concentration was determined using the the Lowry assay with BSA as a standard. Thymidylate synthase specific activity determined using the ³H release method as described in methods and materials. Similar results were obtained in 3 separate experiments.

^b Sample was taken approximately 5 hours after the peak of thymidylate synthase activity observed in figure 1.

^c Sample was taken 3.5 hours after sample b.

^d Sample was taken 17 hours after sample b.

was confirmed by the APE (N-acetyl-phenylalanine¹⁴C-B-naphtyl ester) agar overlay test (Zubenko et al. 1982) (data not shown). During the transition from log phase growth to stationary phase, the rate loss of thymidylate synthase activity was indistinguishable in strains 2180 (pep4-3) and AH22 (PEP4) (fig. 1A). Therefore thymidylate synthase activity disappeared by a PEP4 independent process and inactivation probably did not occur by a vacuolar based proteolytic event.

Thymidylate synthase activity following the refeeding of stationary phase cells

Is protein synthesis required to regain thymidylate synthase activity which was lost during stationary phase? To address this question, increases in thymidylate synthase were followed when stationary phase cells were resuspended in fresh media with and without cycloheximide. A stationary phase culture of strain AH22-pYT760-ryp3 which had lost over 90% of its thymidylate synthase activity was resuspended in fresh media with and without 100 µg/ml of cycloheximide as described in materials and methods. Thymidylate synthase activity, β-galactosidase activity, protein content, and the percentage of unbudded cells were followed during the subsequent growth of the culture. Within 4 hours, thymidylate synthase activity increased approximately 75

fold while β -galactosidase activity and total protein content increased by about 30% (fig. 2). Furthermore, increases in thymidylate synthase activity preceded entry into S phase as determined by the appearance of small budded cells (fig. 2). The addition of cycloheximide immediately after refeeding completely blocked the increases in thymidylate synthase activity, β -galactosidase activity, and total protein (fig. 2).

Because cycloheximide is an inhibitor of protein synthesis, these results are consistent with the notion that thymidylate synthase was irreversibly lost during stationary phase.

The decrease in thymidylate synthase activity as cells entered stationary phase could have been due to the presence of a soluble inhibitor or the disappearance of a soluble activator. To address this question, cell free extracts were prepared from cells at various stages of growth. Mixing extracts prepared from exponentially growing cells (high activity) with extracts prepared from cells harvested at various times during the transition from log to stationary phase (low activity) resulted in additive levels of activity (table 4). Therefore yeast cell extracts did not contain diffusible soluble effectors capable of activating or inhibiting thymidylate synthase activity. Although mixing experiments like these need not reflect in vivo conditions, the results are nonetheless

FIGURE 2

Thymidylate synthase activity, percentage of unbudded cells, total protein, and β -galactosidase activity during outgrowth from stationary phase. A stationary phase culture of strain AH22-pYT760-ryp3 incubated until over 90% of the thymidylate synthase activity disappeared was harvested and resuspended at 9×10^6 cells/ml in fresh prewarmed 2x YNBD media with (open symbols) and without (closed symbols) 100 μ g/ml of cycloneximide. At various times thereafter samples were harvested and stored at -80°C for later assay.

A: relative levels of thymidylate synthase activity as determined by ^3H release assay of Brij 35 permeabilized cells.

B: relative total protein per ml of culture following resuspension in fresh media.

C: relative β -galactosidase activity per ml of culture following resuspension in fresh media.

The results shown were obtained in a total of 3 separate experiments.

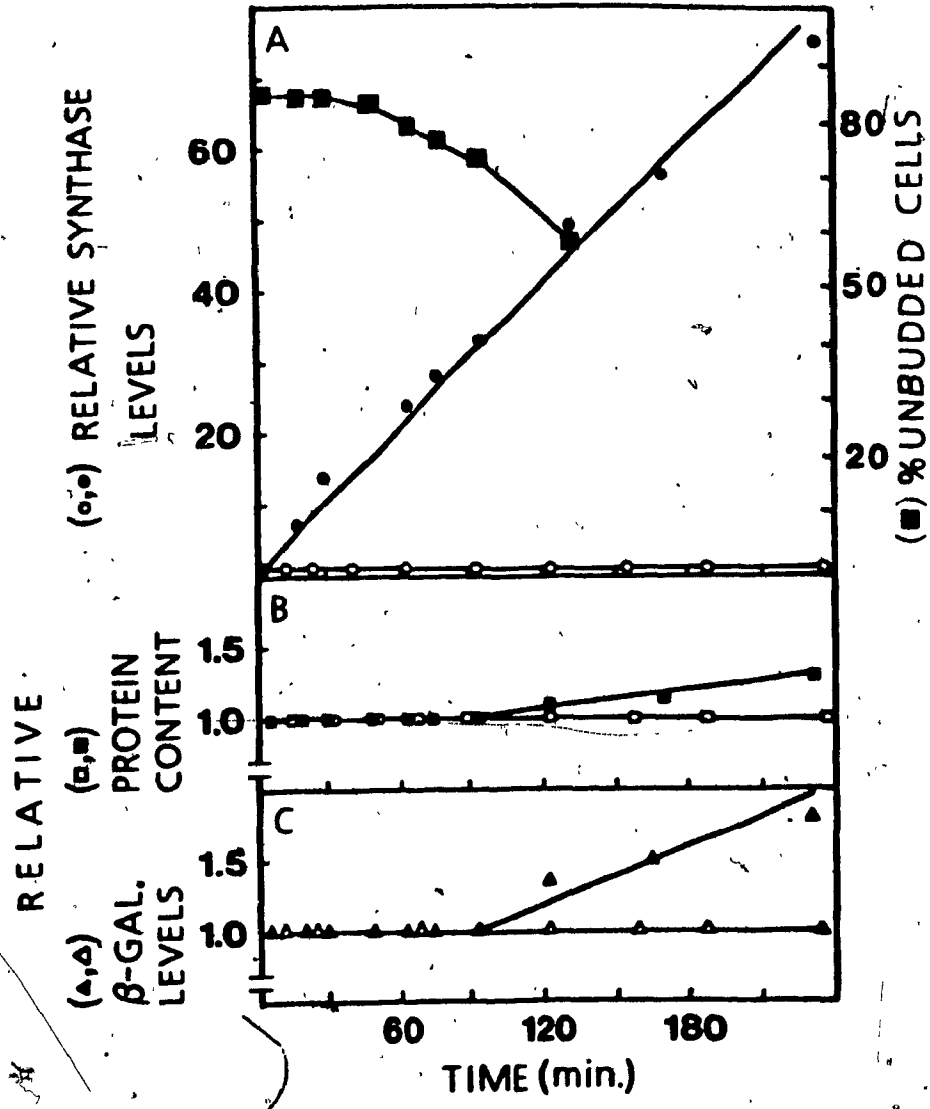


Table 4

Effect on thymidylate synthase activity levels upon mixing high and low activity extracts

Extract mixtures ^a	Ratios	Observed units	Expected units	Observed/Expected
A+B	1 : 2.1 ^b	8.6	8.2	1.05
A+C	1 : 0.7 ^c	4.7	4.7	1.00
	1 : 1.5 ^c	6.8	5.9	1.15
	1 : 2.1 ^b	7.0	6.7	1.05
	1 : 2.9 ^c	7.9	7.3	1.08
A+D	1 : 2.1 ^b	4.3	4.0	1.08

^aCell extracts were mixed in various proportions, allowed to stand on ice for 10 minutes, and thymidylate synthase activities determined by the ³H release assay. This experiment was not repeated. Extracts used were the same as those used in the experiments presented in table 3. A:exponential phase, B:early stationary phase, C:mid stationary phase, and D:late stationary phase.

^b1 corresponds to 0.19 mg of soluble protein.

^c1 corresponds to 0.17 mg of soluble protein.

consistent with thymidylate synthase activity being irreversibly lost.

The effect of α -factor induced G1 arrest on the levels of thymidylate synthase

Previous results had shown that thymidylate synthase activity and its specific mRNA accumulated in a periodic fashion near the beginning of S phase of the yeast cell cycle (Storms et al. 1984). Therefore cells arrested in G1 should have low levels of thymidylate synthase specific mRNA and show a reduced rate of thymidylate synthase synthesis. As a preliminary test of this hypothesis, strain AH22 was arrested in G1 using α -factor (see materials and methods). Two samples were harvested, one at $t=0$ and one 2 hours later when 92% of the cells were unbudded indicating that the culture had been arrested in G1. Crude cell free extracts were made and used to determine thymidylate synthase specific activity. The results obtained show a 50% reduction in the specific activity of thymidylate synthase from 23.1 $\mu\text{U}/\text{mg}$ protein in asynchronous cells (45% unbudded cells) to 12.1 $\mu\text{U}/\text{mg}$ protein in α -factor arrested cells.

To determine if loss of thymidylate synthase activity contributed to the decrease in the specific activity of thymidylate synthase, the levels of activity were followed per ml of culture during α -factor arrest.

Strain AH22 was treated with α -factor and samples were harvested at $t=0$ and at 30 minute intervals thereafter. These samples were used to measure thymidylate synthase activity per ml of culture using Brij 35 to permeabilize the cells. After the addition of α -factor, there was a progressive decrease in the thymidylate synthase level per ml of culture (fig. 3A). Over 20% of the total activity was lost after 2.5 hours of treatment.

Thymidylate synthase levels increased dramatically at about 3 hours, when the culture spontaneously released from α -factor induced G1 arrest as shown by the decrease in the percentage of unbudded cells (typical results obtained in several experiments). This suggested that α -factor arrest was responsible for the observed loss of thymidylate synthase activity.

Treatment with α -factor caused a marked increase in cell size which can be attributed to the fact that cell cycle progression was arrested while synthesis of most protein and RNA species continued. In fact, α -factor arrest does not affect the rate of protein and RNA accumulation (Throm and Duntze 1970). To show that this is also true for the conditions used here, strain AH22-pYT760-ryp3 was treated with α -factor and samples were harvested at $t=0$ and at subsequent time intervals to determine both β -galactosidase activity and total protein content. The results of such an experiment (figure 3B)

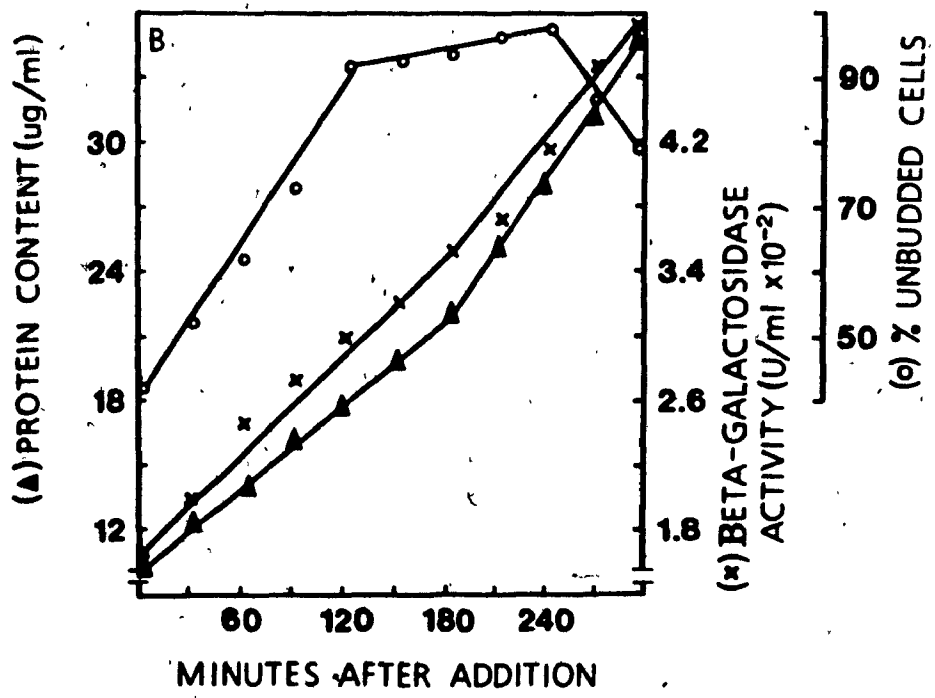
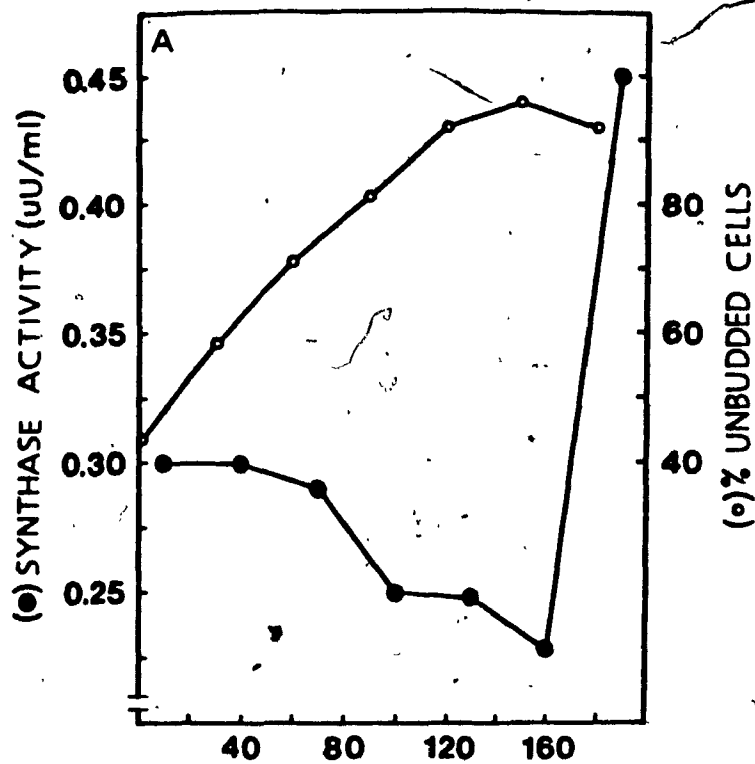
FIGURE 3

Effect of α -factor on thymidylate synthase activity, protein content, and β -galactosidase activity. Cultures were grown overnight to cell density of about 5×10^6 cells/ml. Cells were resuspended in α -factor containing media at $t=0$ and samples were collected at the times indicated.

A, thymidylate synthase activity, per ml of culture, following addition of α -factor to a culture of AH22. At $t=0$ there were about 2×10^6 cells/ml. The experiment was repeated two more times with similar results.

B, β -Galactosidase activity and protein content, per ml of culture, following addition of α -factor to a culture of RS290. At $t=0$ there was about 1×10^6 cells/ml.

Experiments were not repeated.

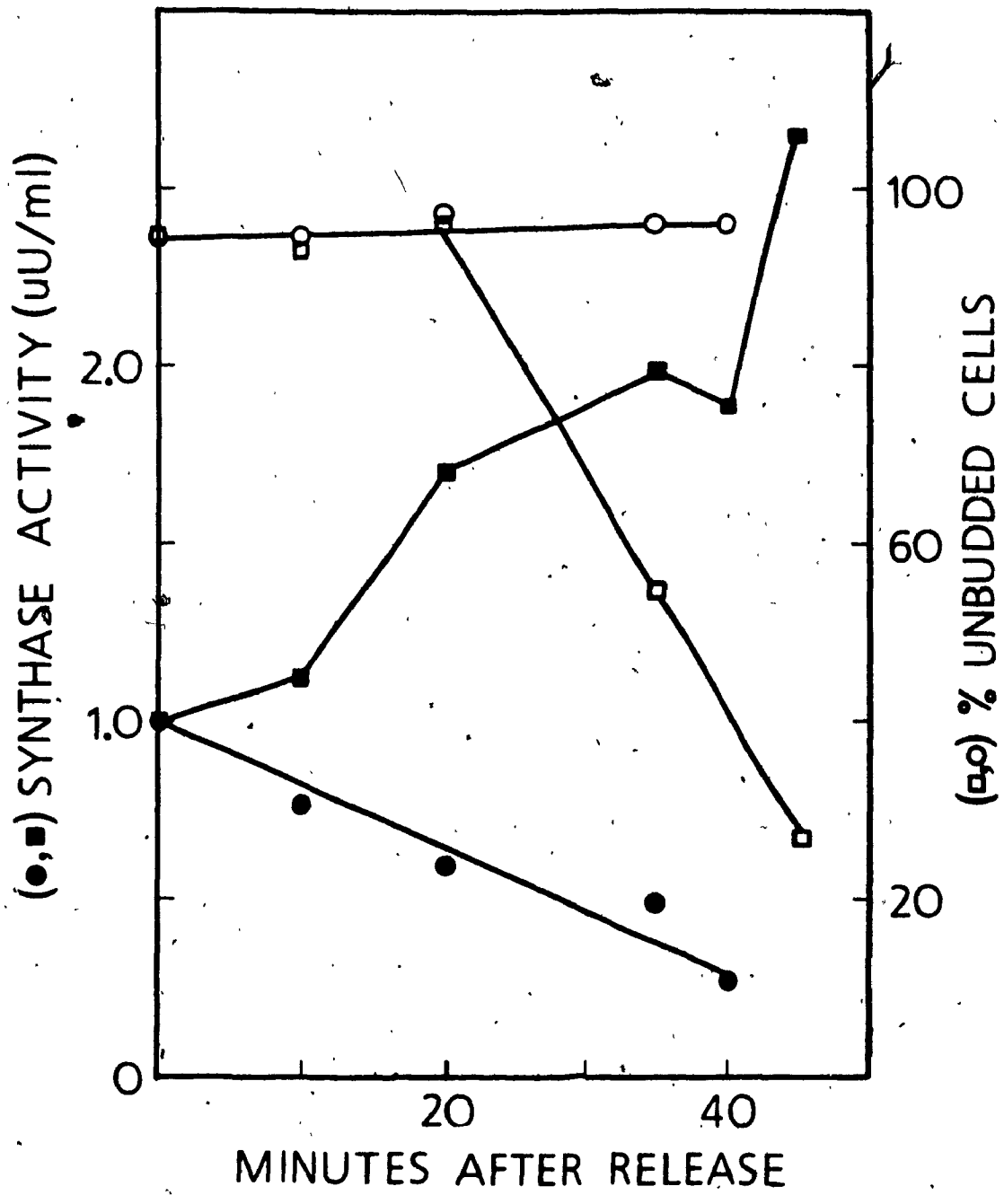


showed that both protein content and β -galactosidase activity per ml of culture continued to increase even after prolonged periods of G1 arrest. Furthermore, the release from G1 arrest caused no change in the rate of accumulation of both β -galactosidase activity and total protein. This is unlike the results obtained for thymidylate synthase. Taken together these results suggested that thymidylate synthase is unlike the bulk of cellular proteins. Its rate of accumulation did change in response to α -factor induced G1 arrest. In fact, because activity of the culture actually decreased, inactivation played a role in determining activity levels.

The results depicted in figure 3A showed that strain AH22 spontaneously released from pheromone arrest about 2.5 hours after α -factor was added. Release from arrest occurs because a mating type cells metabolize α -factor (Bucking-Thom et al. 1973). Release from cell cycle arrest, shown by a significant increase in the percentage of cells with buds, resulted in a dramatic increase in TMP1 encoded mRNA (Storms et al. 1984) and thymidylate synthase activity (fig. 3A, and Storms et al. 1984). The concomitant increase in thymidylate synthase mRNA and activity suggested that new synthesis is responsible for the burst in thymidylate synthase activity. Further evidence that the increase resulted from a burst of synthesis is shown in figure 4. Here a culture

FIGURE 4

Effect of cycloheximide on the increase in thymidylate synthase activity following release from α -factor arrest. Culture of strain AH22 was treated with α -factor as described in materials and methods. When the culture reached over 90% unbudded morphology, α -factor was quickly removed by filtration and the cells were resuspended in fresh 0.1% media with and without 100 μ g/ml of cycloheximide. Following release, samples were taken at intervals and thymidylate synthase and bud morphology determined. Thymidylate synthase activity (closed symbols), bud morphology (open symbols). The culture without cycloheximide is represented by squares, and the culture with cycloheximide by circles. Experiment was not repeated.



of strain AH22 was arrested in G1 with α -factor. When greater than 90% of the cells were unbudded, α -factor was quickly removed and the cells resuspended in two flasks of fresh 0.1% media, one with 100 μ g/ml of cycloheximide and one without cycloheximide. The results showed that cycloheximide prevented the accumulation of thymidylate synthase activity following removal of α -factor.

Levels of thymidylate synthase increased in response to an increase in the cell doubling time

Steady state levels of thymidylate synthase were measured in strain AH22 grown in 3 separate media. Table 5 shows that the specific activity of thymidylate synthase increased and the generation times of AH22 decreased with increasingly rich media. These results suggested that yeast cells somehow modulate thymidylate synthase activity levels in response to alterations in the generation times (see discussion).

Table 5

Thymidylate synthase levels in response to an increase in growth rate^a

growth media	generation time (min)	synthase activity	
		$\mu\text{U}/\text{mg}$	$\mu\text{U}/10^8$ cells
2xYNBD			
(minimal)	150	26	44.8
YEPD			
(rich)	120	32.3	75.7
0.1%			
("super-rich")	90	37.3	ND ^b

^a Exponentially growing cultures of strain AH22 were generated in 3 different growth medias. At various time intervals during exponential growth, the turbidity of the culture was measured (OD 600 nm) to determine cell doubling times. Samples were also harvested to determine the steady state levels of thymidylate synthase. Results shown are similar to the results obtained in 2 more experiments.

^b ND, not determined.

SECTION 2: Genetic approach

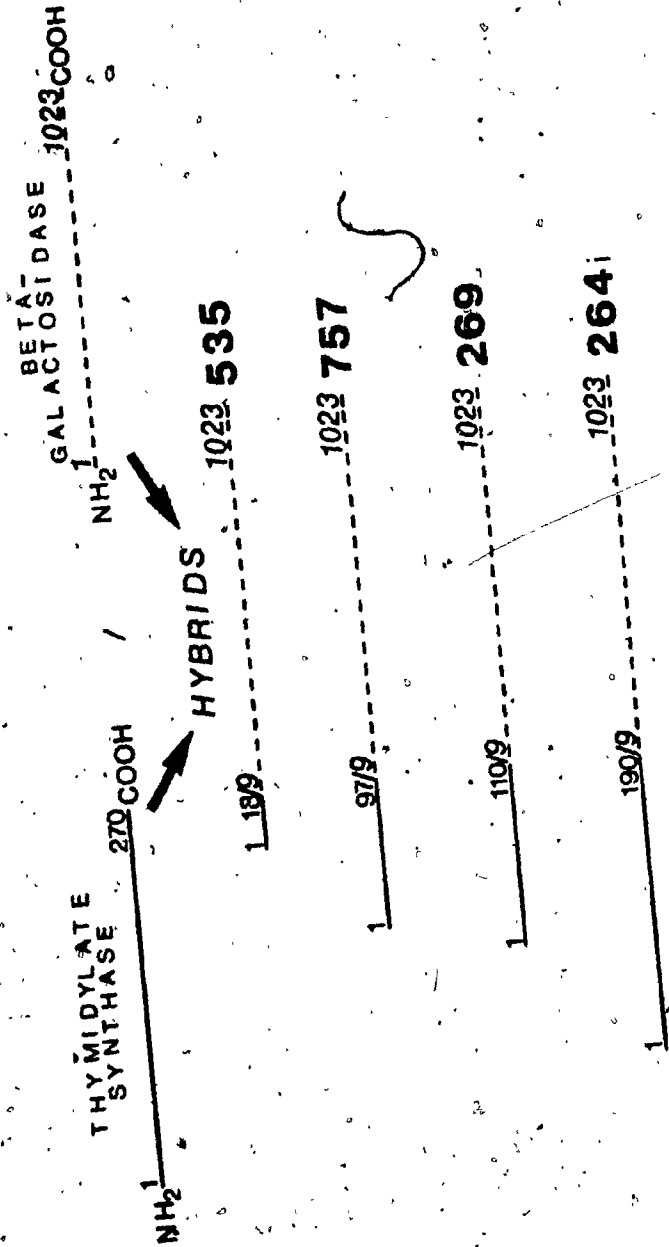
Description of fusions

We next used a genetic approach to further look at the factors governing the levels of thymidylate synthase activity. This approach involved studying a series of 4 gene fusions between the TMP1 gene and the E. coli lacZ structural gene. These fusion genes encode fusion proteins which contain a constant portion of the lacZ structural information fused in frame (for translation) to increasing amounts of N-terminal TMP1 structural information. Fig. 5 is a schematic representation of the different hybrid proteins encoded by the TMP1 - LacZ gene fusions. Since the first 8 amino acids of LacZ are lacking, the translational start site (ATG) in the fusions are supplied by the TMP1 gene. In addition, the 5' end of the TMP1 gene is responsible for promoting transcription of the fusion genes. The construction of the gene fusions will be described elsewhere (R.W. Ord, PhD thesis in preparation, Concordia University, and R.W. Ord, et al., manuscript in preparation). For the purpose of this thesis, I will only describe certain features of these fusions which are essential for the these experiments.

All the fusion genes are carried on identical plasmids, the only difference being the variable amount of

FIGURE. 5

Schematic representation of the different thymidylate synthase β -galactosidase hybrid proteins. The hybrid proteins consist of an increasingly large portion of thymidylate synthase (full lines) at the amino terminal and a constant portion of β -galactosidase (dashed line) at the carboxy terminal. Also depicted, the number of thymidylate synthase and β -galactosidase amino acids in the native and hybrid proteins.



TMP1 structural information fused to lacZ (table 2). The plasmids contain a portion of the 2 μ circle and the entire LEU2 gene. This allows for replication and selection in yeast. A total of four different protein fusions were studied. These are encoded by plasmids pRS535, pRS757, pRS269, and pRS264i which contained the first 13, 97, 110, and 190 amino acids of thymidylate synthase respectively (table 6 and fig. 5).

All the TMP1 - LacZ hybrid genes produce hybrid proteins containing a portion of the N-terminal amino acids of thymidylate synthase. As a control, we made use of plasmid pYT760-ryp3 which contained a fusion gene consisting of the LacZ structural gene expressed from an unknown yeast promoter (ryp-3). The rest of the plasmid is otherwise identical to the other fusion containing plasmids. The β -galactosidase hybrid protein expressed from plasmid pYT760-ryp3 contains no thymidylate synthase amino acids.

Expression of the TMP1-LacZ fusions

The plasmids containing the fusion genes were introduced into yeast strain AH22 by the technique of transformation. The transformants were selected for and maintained by their ability to grow without exogenously supplied leucine (see materials and methods). To determine the levels of β -galactosidase expressed from the various

fusion genes, cultures of the fusion bearing strains were grown to mid-exponential phase as defined in methods and materials and samples collected. These samples were used to measure the levels of β -galactosidase and thymidylate synthase. The values obtained are shown in table 6.

Thymidylate synthase activity can be seen to be similar in all strains suggesting that the plasmids did not effect expression of the chromosomal TMPl gene. In contrast, β -galactosidase activity in the different fusion containing strains varied by as much as 20 fold. Examination of the data showed that the levels of β -galactosidase activity increased with increasing amounts of TMPl structural information present in the fusion gene.

β -galactosidase activity during the transition from log growth to stationary phase

Thymidylate synthase activity was rapidly lost as log phase cultures entered stationary phase (fig. 1). The loss of thymidylate synthase activity is presumably due to a portion of its amino acid sequence being recognized by an "inactivation machinery". If this was so, β -galactosidase hybrid proteins containing the thymidylate synthase "inactivation sequence" would render β -galactosidase activity unstable in a manner similar to thymidylate synthase. In order to test this hypothesis,

Table 6

Thymidylate synthase and β -galactosidase activities of strain AH22 transformed with different plasmids

plasmid	number of TS amino acids in fusion ^a	generation time (min) ^b	<u>Activities</u> ^c	
			β -gal	synthase
none	--	150	<0.01	5.4
PRS264i	190	160	8.1	4.6
PRS269	110	160	1.3	4.9
PRS757	97	ND ^d	0.7	ND
PRS535	13	150	0.4	4.1
PRS306 ^e	110	150	0.04	ND
pYT760- ryp3 ^f	--	150	1.2	--

^aAll fusion proteins have the last 1005 amino acids of the native β -galactosidase protein (1013 amino acids).

^bGeneration time was determined during growth on 2x YNBD media. Leucine was omitted to maintain plasmid selection.

Table 6 (cont'd)

^c Samples from exponentially growing cultures (in 2x YNBD media) of the different strains were harvested at an OD 600 of between 0.2 and 0.3 or at 70 Klett units. β -galactosidase and thymidylate synthase activities are expressed per ml of culture of 1 OD unit at 600 nm. β -galactosidase and thymidylate synthase activities were determined as described in materials and methods. Results were similar in 3 separate experiments.

^d Not determined.

^e Plasmid pRS306 must integrate into the yeast genome since it lacks an origin of replication. Strain RS458 contains 1 copy of plasmid pRS306 integrated into the yeast genome at the leu2 site in strain AH22 (see text).

^f Plasmid pYT760-~~pyp3~~ contains a lacZ fusion with no TMP1 information but is otherwise identical to the others (see text).

strains AH22-PRS535, AH22-PRS269, and AH22-PRS264i were grown to late exponential phase, and sampling was then initiated and continued for a further 29 hours.

As the cultures entered stationary phase, β -galactosidase activity encoded by the 2 largest fusion genes disappeared, at a rate similar to that observed for thymidylate synthase itself (compare fig. 1A with fig. 6 upper and middle panels). In contrast, β -galactosidase activity encoded by the smallest fusion gene continued to increase during late stationary phase (fig. 6 lower panel). Taken together, these results suggested that the information between amino acids 13 and 110 may serve as a signal which lets the cell know that proteins containing it are to be rapidly turned over during the transition from log growth to stationary phase.

Similar experiments, measuring β -galactosidase activity during the transition from log growth to stationary phase were carried out using the pep4-3 mutant strain 4A carrying plasmid PRS269. β -galactosidase activity was seen to decay when logarithmically growing cultures of strain 4A-PRS269 (pep4-3) entered stationary phase in a similar manner to the observed loss in strain AH22-PRS269 (PEP4 ; Sym Davis, personal communication). This suggested that the loss of β -galactosidase activity during entry into stationary phase, like the loss of thymidylate synthase activity, did not depend on vacuolar

FIGURE 6

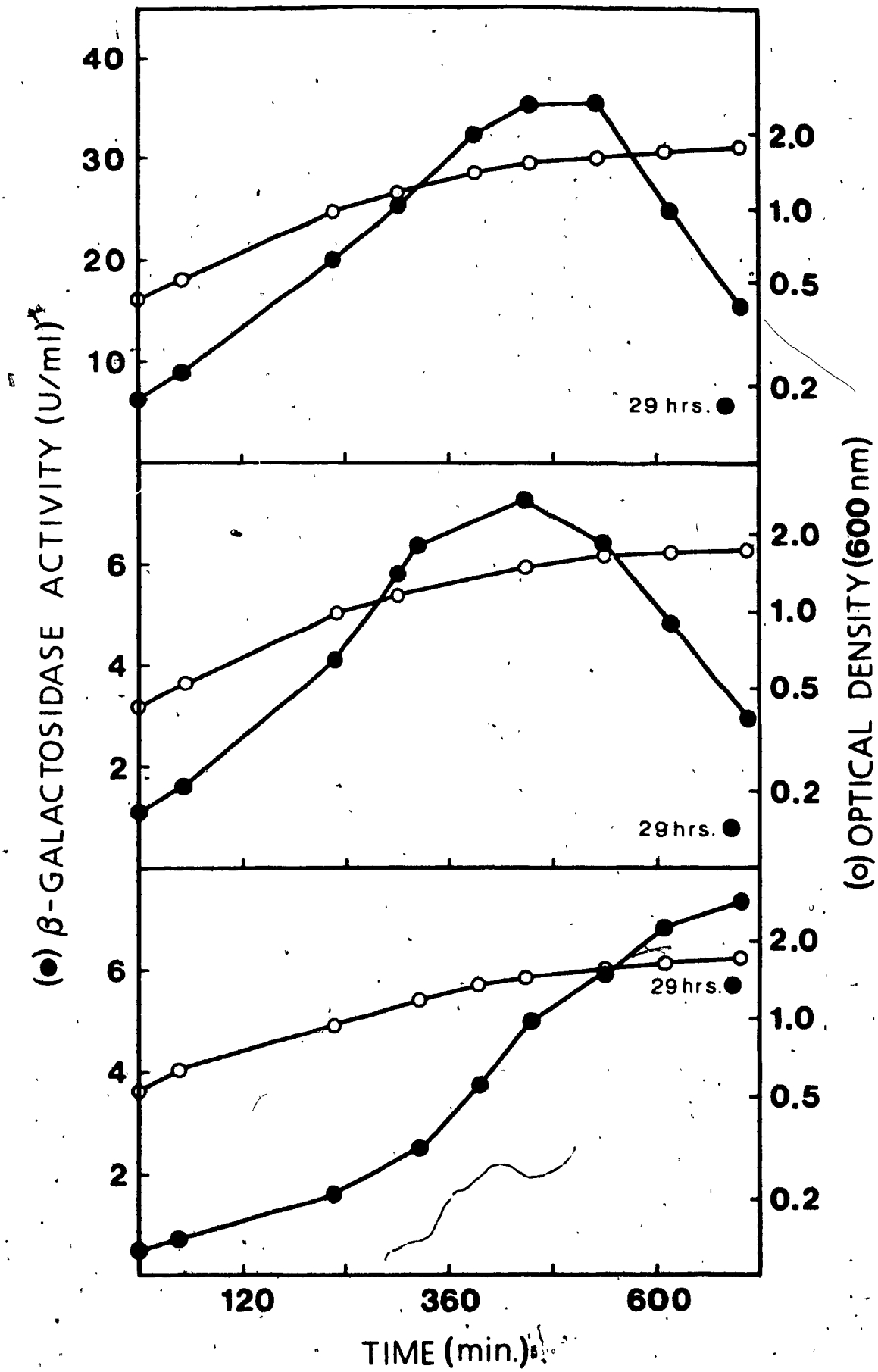
β -galactosidase activity and growth curve during the transition from logarithmic growth to stationary phase. Samples were harvested from exponentially growing cultures, at intervals, until stationary phase was reached. These samples were used to determine both β -galactosidase activity and culture density (OD 600 nm). The final samples were taken 29 hours after the first samples.

Upper panel: AH22-pRS264i (190 thymidylate synthase amino acids)

Middle panel: AH22-pRS269 (110 thymidylate synthase amino acids)

Lower panel: AH22-pRS535 (13 thymidylate synthase amino acids)

Similar results were obtained in a total of 3 separate experiments.



based proteases.

β -galactosidase activity in response to α -factor induced G1 arrest

Thymidylate synthase activity per ml of culture has been shown to decrease when cells are arrested in G1 with α -factor (fig. 3A). The same experiment was performed with the strains bearing the different TMP1 - lacZ fusions. When these strains are arrested in G1 by α -factor, β -galactosidase activity of the 2 largest fusion proteins decreased while the activity of the smallest fusion protein continued to increase (fig. 7). These results further confirmed the localization of an "inactivation signal".

Levels of β -galactosidase in response to cycloheximide

In order to obtain the actual rate loss of β -galactosidase activity for the different fusion proteins, exponentially growing cultures were treated with cycloheximide (an inhibitor of protein synthesis). Cultures of strains harboring the various fusion genes were grown to an OD 600 of around 0.3 and 100 μ g/ml of cycloheximide was added. Samples were harvested before the addition of cycloheximide ($t=0$) and at intervals after treatment. After the addition of cycloheximide, β -galactosidase activity of fusion proteins 264i, 269, and

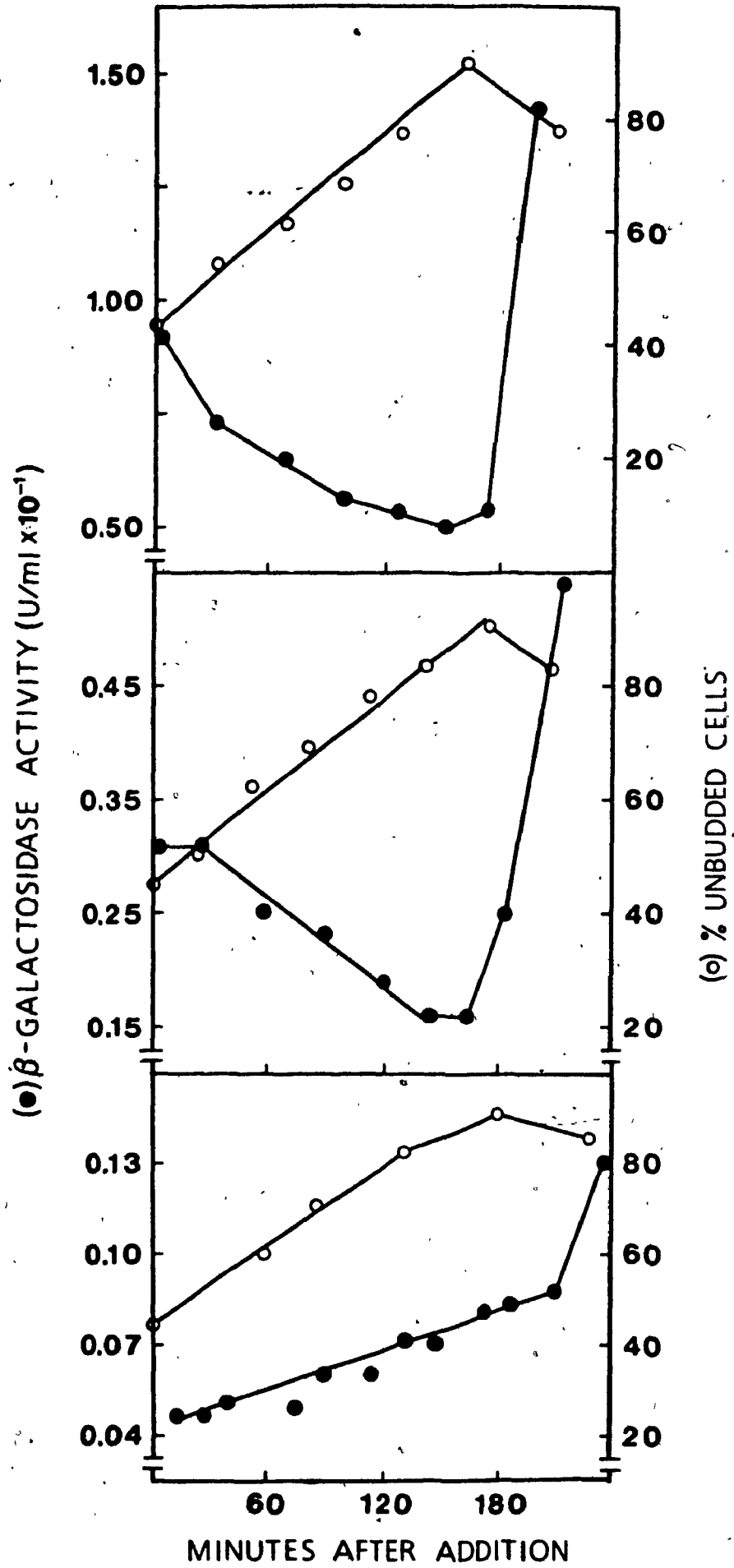
FIGURE 7

Effect of α -factor on β -galactosidase activity and on bud morphology. Exponentially growing cultures were allowed to reach a cell density of about 5×10^6 cells/ml. The cultures were then resuspended in α -factor containing media at about 2×10^6 cells/ml. During the subsequent growth, samples were harvested at intervals. These samples were later used to determine β -galactosidase levels and bud morphology.

Upper panel: AH22-pRS264i (experiment was repeated twice)

Middle panel: AH22-pRS269 (experiment was repeated twice)

Lower panel: AH22-pRS535 (experiment was repeated 4 times)



757 decayed with an initial half life of 80, 40, and 40 minutes respectively (fig. 8A, B, and C). In contrast, β -galactosidase activity from the smallest fusion protein (535) was completely stable for over 5 hours after the addition of cycloheximide (fig. 8D). The β -galactosidase activity encoded by the control fusion on pYT760-ryp3, which contained no TMP1 information, was also stable (fig. 8E). These results showed that fusing the N-terminal amino acids of thymidylate synthase to β -galactosidase renders the normally stable β -galactosidase activity unstable in yeast.

We wanted to determine the effect of decreasing the level of β -galactosidase on the rate loss of activity. To accomplish this, I made use of strain RS458, which was constructed by Lydia Lee. This strain contained a single copy of a fusion gene encoding a β -galactosidase protein, identical to 269, integrated at the leu2 locus (L. Lee, personal communication). It was found that this strain RS458, produced approximately 30 times less β -galactosidase activity than strain AH22-pRS269 (table 5). Treatment of an exponentially growing culture of strain RS458 with 100 μ g/ml of cycloheximide resulted in β -galactosidase activity being lost at a rate which was indistinguishable from that found with AH22-pRS269 (compare fig. 8B with 8F). It can therefore be concluded that levels of β -galactosidase encoded by pRS269 did not

FIGURE 8

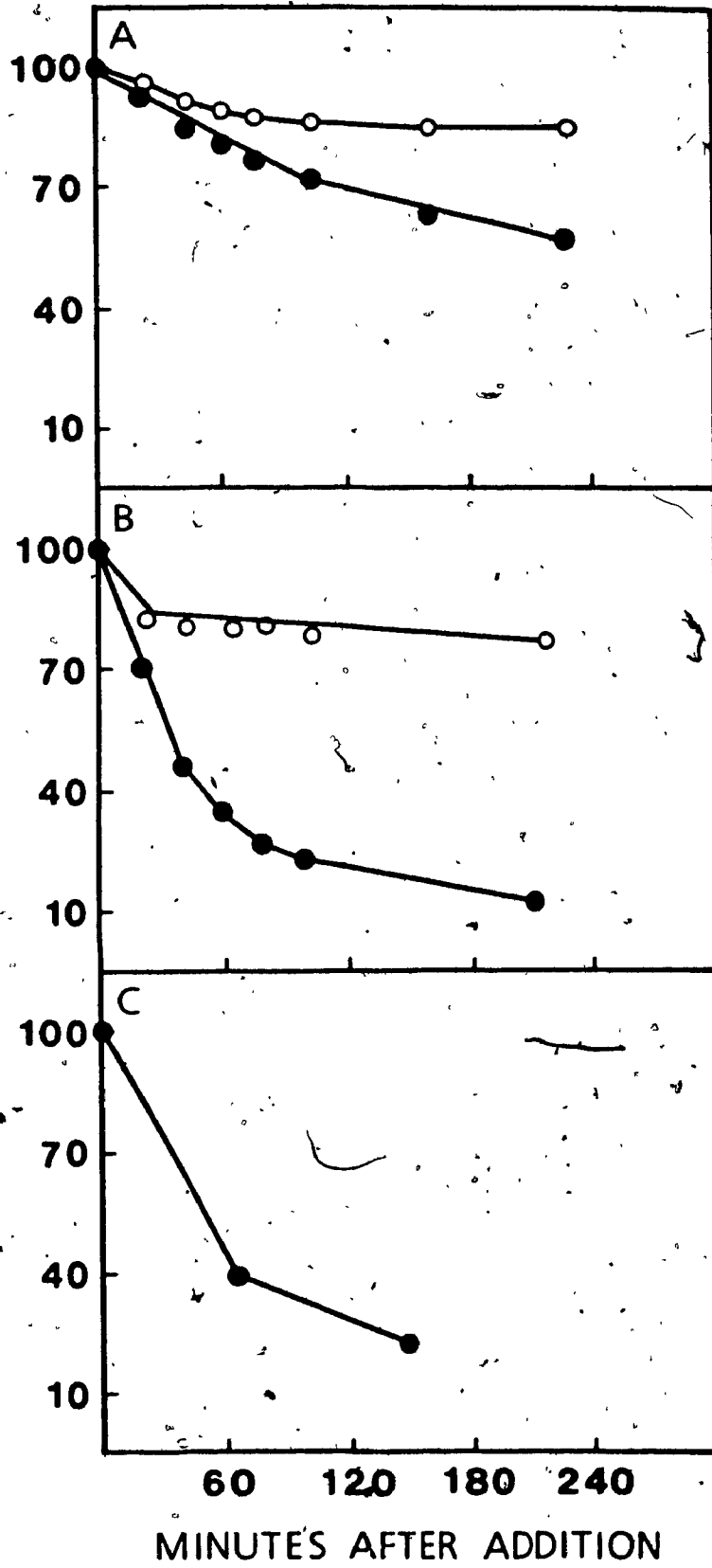
Effect of cycloheximide alone and in combination with azide on β -galactosidase activity. Exponentially growing cultures were generated as described in materials and methods. When the culture density reached about 1×10^7 cells/ml, cycloheximide (100 μ g/ml) was added. For strains AH22-pRS264i and AH22-269, exponentially growing cultures were separated into 2, one culture received cycloheximide alone and the other received both cycloheximide and Na azide (100 μ g/ml). Following the additions, 1 to 10 ml culture samples were harvested and stored at -80°C as described in materials and methods. β -galactosidase activity per ml of culture was determined using Brij 35 to permeabilize the cell samples.

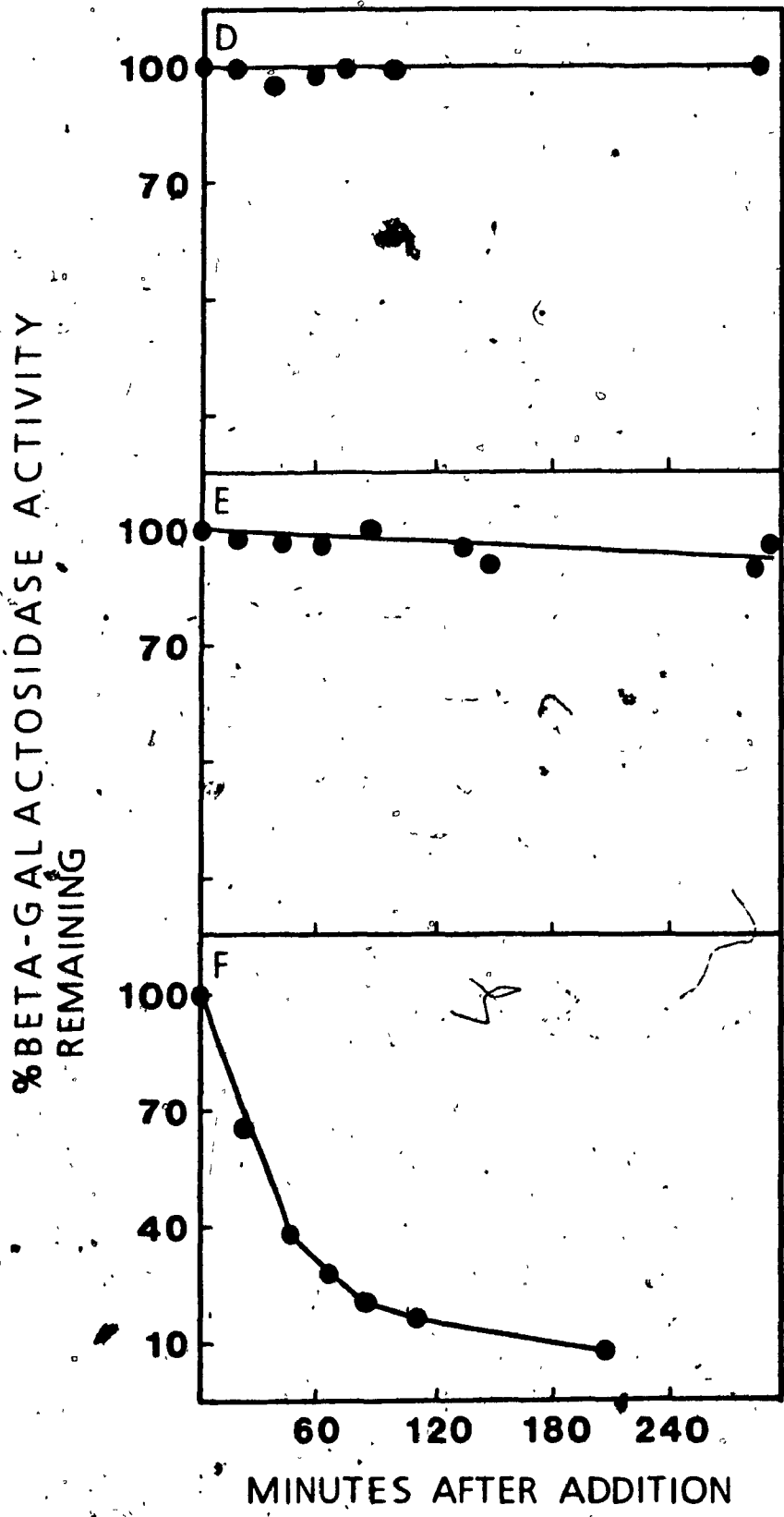
With cycloheximide alone (closed circles)

With cycloheximide and Na azide (open circles)

A: AH22-pRS264i (repeated twice); B: AH22-pRS269 (repeated 6 times); C: AH22-pRS757 (repeated once); D: AH22pRS535 (repeated once); E: AH22-pYT760-ryp3 (repeated once); F: RS458 (not repeated).

%BETA-GALACTOSIDASE ACTIVITY
REMAINING





saturate the "inactivation machinery" and did not influence the rate of decay.

Energy dependent inactivation of β -galactosidase activity

The in vivo degradation of proteins was shown to have an absolute requirement for energy in many organisms (reviewed in Golberg and St John 1976). That this is also true in yeast was shown by Lopez and Gacendo (1979). To determine the energy requirements for the loss of β -galactosidase, two potent inhibitors of cellular energy production were used, cyanide and azide. Both compounds have been shown to effect energy production by interfering with the flow of electrons along the electron transport chain of mitochondria (Lardy and Ferguson 1969). Exponentially growing cultures of strains AH22-264i and AH22-269 were allowed to reach an OD 600 of around 0.3. At this point each culture was separated into 2 flasks, one was treated with cycloheximide while the other received cycloheximide and 0.1 mg/ml of either Na azide or K cyanide. Samples were harvested prior to the additions and at intervals thereafter and used to determine levels of β -galactosidase. Treatment of cultures with cycloheximide alone resulted in the loss of activity (fig. 8A, B, and fig. 9). The concomitant addition of azide (fig. 8A and B) or of cyanide (fig. 9) with cycloheximide greatly decreased the loss of β -galactosidase activity.

FIGURE 9

Effect of cycloheximide and of cycloheximide in combination with cyanide on the levels of β -galactosidase of strain AH22-PRS269 grown with different carbon sources. Exponentially growing cultures of strain AH22-PRS269 were generated in either 2x YNB media with glucose or in YEP media with glycerol as described in materials and methods. When the culture densities reached 1×10^7 cells/ml, the cultures were separated into 2. One culture received cycloheximide alone and the other culture received both cycloheximide and K cyanide. Just prior to the additions ($t=0$) and at intervals thereafter, culture samples were harvested to determine β -galactosidase levels.

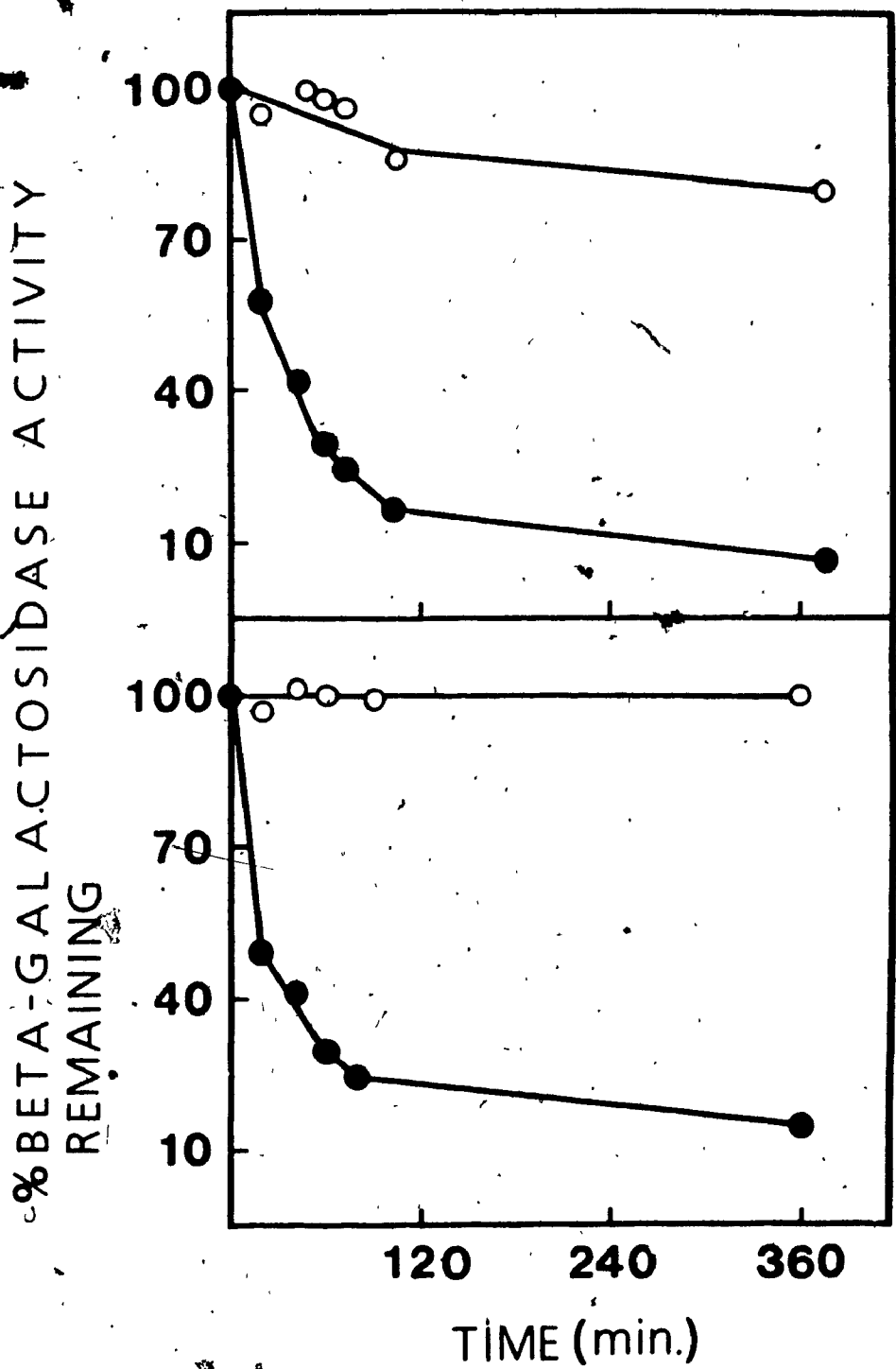
Cycloheximide alone (closed circles)

Cycloheximide and cyanide (open circles)

Upper panel: AH22-PRS269 grown in YNB with glucose

(repeated 6 times)

Lower panel: AH22-PRS269 grown in YEP with glycerol (not repeated)



As observed in figures 8 (A and B) and 9 (upper panel), the addition of azide or of cyanide did not completely prevent the loss β -galactosidase activity which occurred after the addition of cycloheximide. Azide and cyanide inhibit ATP production by blocking oxidative phosphorylation (Lardy and Ferguson 1969). Yeast cells are capable of growth with a fermentable carbon source (i.e. glucose) in the absence of oxidative phosphorylation (reviewed in Dujon 1981). Therefore, azide or cyanide may not totally inhibit energy production in yeast cells growing on glucose. This may explain the small loss of β -galactosidase activity after the addition of cyanide or azide to cells growing on glucose. As a preliminary test of this hypothesis, the effects of cyanide were examined in yeast cells growing with a non-fermentable carbon source (glycerol). Because strain AH22-269 could not grow on minimal media with glycerol as the sole energy source, we had to use rich media (YEP) to obtain aerobic growth. It should be noted that an energy source (i.e. glucose or glycerol) must be added to YEP media to obtain cell growth (T. Downing, pers. comm.).

Cycloheximide alone or cycloheximide with cyanide was added to a culture of strain AH22-pRS269 which was exponentially growing in rich media supplemented with glycerol as the sole energy source (YEPG). Prior to the treatments, and at time intervals thereafter culture

samples were harvested and their levels of β -galactosidase determined. Figure 9 shows that the simultaneous addition of cyanide with cycloheximide completely protects β -galactosidase activity in cells growing on glycerol (YEPG) but not in cells growing on glucose (2x YNBD). Taken together these results show that the loss of β -galactosidase activity is energy dependent.

SECTION 3: Levels of thymidylate synthase¹¹ in response to alterations in the dosage of the TMP1 gene

Description of plasmids

The availability of the cloned TMP1 gene (Taylor et al. 1982) allowed a detailed study correlating levels of thymidylate synthase in response to alterations in the gene copy number. In order to accomplish such a study, the original TMP1 containing plasmid was used. This plasmid, pTL1, contains the TMP1 gene on a 10 Kb Hind III to Hind III fragment of genomic yeast DNA (Taylor et al. 1982). The salient features of this plasmid include the entire LEU2 gene and a portion of the 2 μ circle to allow for selection and replication respectively (table 2). That this plasmid contained the yeast gene coding for thymidylate synthase (TMP1) has been previously shown (Taylor et al. 1982; Storms et al. 1984). Plasmid pTL1' is identical to plasmid pTL1 except that the 10 Kb Hind III to Hind III fragment, containing the TMP1 gene has been inverted relative to the vector sequence. Plasmids like pTL1' and pTL1 are usually maintained at between 10 and 35 copies per cell (Futcher and Cox 1983; Martinez-Arias and M. J. Casadaban 1983; R. W. Ord et al. manuscript in preparation).

I have also used plasmid pRS(cen3)471 (E.M. Calmels, personal communication). This plasmid has an ars

sequence and the LEU2 gene to allow for replication and selection respectively in yeast. In addition plasmid pRS(cen3)471 has the centromere sequence originally isolated from chromosome number 3 (Clark and Carbon 1980). The centromere in association with the chromosomal ars sequence is responsible for maintaining a plasmid at between 1-2 copies per cell (Clark and Carbon 1980).

The effect of increased TMP1 gene dosage on the steady state levels of thymidylate synthase

Plasmids pTL1, pTL1', and pRS(cen3)471 were used to transform strain AH22 to leucine prototrophy. Exponentially growing cultures of all 4 strains (AH22, AH22-pTL1, AH22-pTL1', and AH22-pRS(cen3)471) were generated and the increase in culture turbidity was followed (OD 600nm and Klett units) during the subsequent exponential growth until stationary phase was reached. These readings were used to determine the generation times of the different strains. Samples were harvested from these cultures and used to measure the steady levels of thymidylate synthase per ml of culture and per mg soluble protein. The results of these experiments showed that the presence of the TMP1 containing plasmids had no detectable effect on the generation times of logarithmically growing AH22 (table 7). Measurements of thymidylate synthase activity, by the ³H release

Table 7

Steady state levels of thymidylate synthase in strain AH22 containing different doseages of the TMP1 gene^a

Strain	<u>TMP1</u> copy #	generation time (min) ^b	<u>TS activity</u>		
			μ U/mg	μ U/ml	
			<u>Trial 1/Trial 2</u>		
AH22	1	150	26	25.3	0.9
AH22- pRS (cen3)					
471	2-3	160	84	57.2	1.9
AH22-pTL1	8-15	160	226	279.7	13.7
AH22-pTL1'	8-15	140	283	255	7.1

^a Samples were harvested from exponentially growing cultures and stored at -80°C as described in materials and methods. These samples were then used to determine thymidylate synthase levels per ml of culture (at 70 Klett units) in Brij 35 permeabilized cells or levels were determined per mg soluble protein in crude cell extracts. Results from a third experiment fell within the values shown.

^b Generation times were determined by following increases in turbidity with a Klett and with a spectrophotometer (OD 600 nm). Where necessary, leucine

Table 7 (cont'd)

was omitted from the YNBD media to maintain plasmid selection.

assay, in the 4 strains showed that levels increased in a gene dosage dependent manner (table 7).

When thymidylate synthase levels were determined by the radioactively labelled FdUMP binding assay, a similar increase in activity was seen to occur in response to increased gene dosage (fig. 10). The [6-³H]-FdUMP binding assay is based on the stoichiometric and covalent binding of radiolabelled FdUMP with thymidylate synthase and N⁵,N¹⁰-methylene tetrahydrofolate. Crude cell extracts from AH22, AH22-pTL1, and AH22-pRS(cen3)471 were treated with [6-³H]-FdUMP as described in materials and methods. After ternary complex formation, 25 µg of total protein was boiled in SDS and subsequently separated by SDS-polyacrylamide gel electrophoresis. The resultant Coomassie blue stained gel and the subsequent autoradiograph are shown in figure 10. The results show that in 25 µg of soluble yeast protein there is an increased level of [³H]-FdUMP binding capacity as the dosage of the TMPl gene increased. In addition the autoradiograph showed that the molecular weight of the thymidylate synthase monomer is about 32,000, which agreed with previously published estimates (Bisson and Thorner 1981; Storms et al. 1984).

In order to determine the levels of thymidylate synthase with a variable number of chromosomal copies of the TMPl gene, diploids with 1 and 2 functional copies

FIGURE 10

Gene dosage dependent increase in [6-³H]-FdUMP binding. Cell free extracts were prepared from samples of exponentially growing cultures of strains AH22, AH22-pTL1, and AH22-pRS(cen3)471. These extracts were then treated with [6-³H]F-dUMP in the presence of N5-N10-methyltetrahydrofolate to allow ternary complex formation (Belfort et al. 1983). 25 µg of total soluble protein, from each extract, was then separated on a 12.5% polyacrylamide gel. The ordering on the gel is, from left to right, AH22, AH22-pTL1, and AH22-pRS(cen3)471.

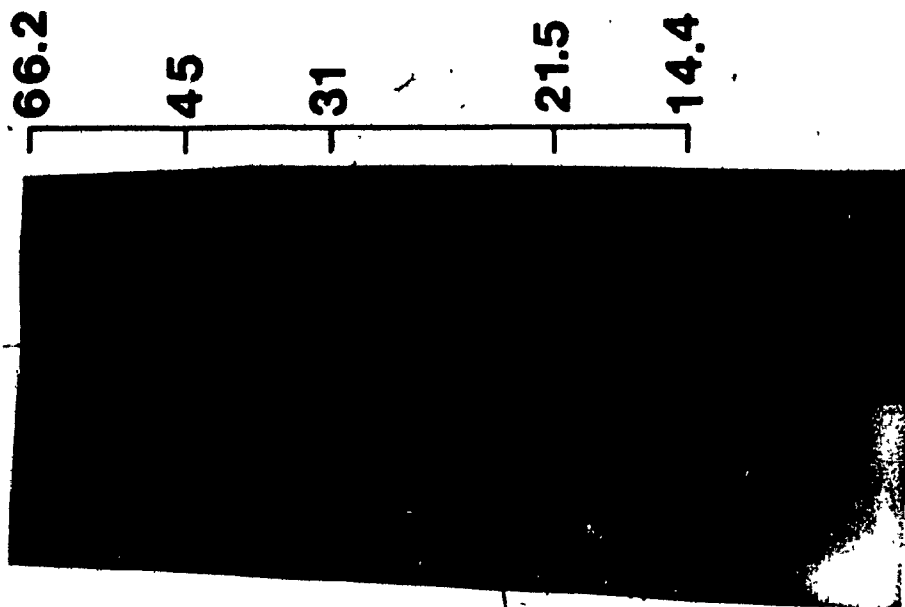
A, the coomassie blue stained gel

B, fluorogram of the gel after 120 days of exposure at -80°C

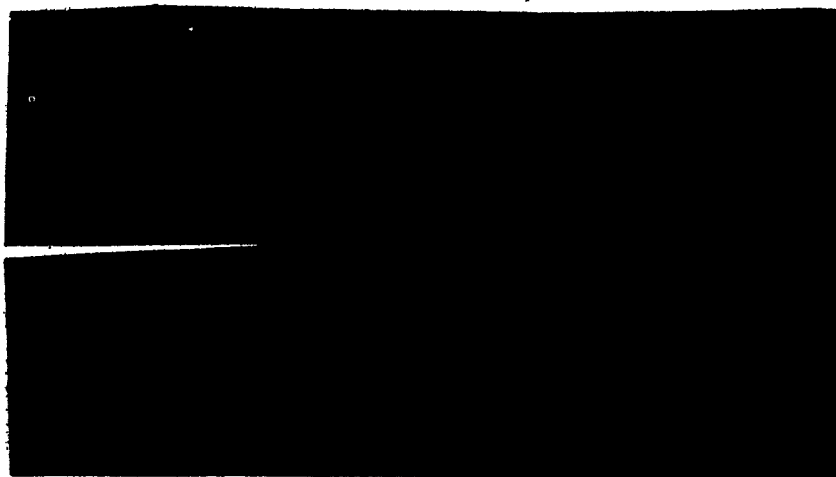
Molecular weight scale (in thousands) is shown.

The experiment was repeated once.

A



B



of the TMP1 gene were constructed. Strain RS422 results from mating haploid strains AH22 and LL20 and has 2 copies of the TMP1 gene, while strain RS421 results from mating haploid strains BB30-3 and LL20 and has 1 functional copy of the TMP1 gene. The complete genotypes as well as the method used to construct the diploids are described in materials and methods.

Exponentially growing cultures of strains RS421 and RS422 were generated to allow the determination of cell doubling times. In addition, cell samples were harvested from both cultures to determine the steady state levels of thymidylate synthase per ml of culture and per mg soluble protein. The generation times of the 2 diploid strains are indistinguishable, but strain RS422 has twice as much thymidylate synthase activity as does strain RS421 (table 8). These results suggested that 50 % of the wild type diploid level of thymidylate synthase activity is sufficient to supply enough dTMP to maintain normal rates of DNA replication.

Is the excess thymidylate synthase activity physiologically functional?

Yeast cells are not normally permeable to phosphorylated compounds such as dTMP orFdUMP (Brendel and Haynes 1972). Mutants (called tup) have been isolated which are permeable to these compounds (Bisson

Table 8

Thymidylate synthase activity in diploids^a

Strain ^b	number of functional <u>TMP1</u> genes	generation time (min) ^c	TS activity	
			μ U/mg	μ U/ml
RS421	1	162	6.2	0.96
RS422	2	168	14.9	2.06

^aSamples were harvested from exponentially growing cultures to determine thymidylate synthase levels per mg of protein in cell free extracts and per ml of culture (at 70 Klett units) in Brij 35 permeabilized cell samples. Results shown here were similar to the results obtained when the experiment was repeated a second time.

^bDiploid strains RS421 and RS422 were contrasted as described in materials and methods.

^cGeneration times were determined by following increases in culture turbidity at OD 600 nm.

and Thorner 1977). These tup mutants can grow with exogenously supplied dTMP and are inhibited by FdUMP. The stoppage of DNA synthesis due to FdUMP in these tup mutants is believed to be due to its inhibitory effect on thymidylate synthase (Bisson and Thorner 1982). This is partly because exogenously supplied dTMP can support growth in the presence of FdUMP. It was therefore reasoned that yeast cells having increased levels of thymidylate synthase (i.e., due to plasmid pTL1) would have an increased resistance to FdUMP. Strain BB30-3 was used to verify this was the case. This strain is leu2⁻, tmp1⁻, and tup⁺, and therefore required exogenously supplied dTMP and leucine for growth. In addition this strain can take up FdUMP.

BB30-3 was transformed to leucine prototrophy with plasmids pTL1 (8-15 copies/cell) and pRS(cen3)471 (1-2 copies/cell). Transformants which could grow without leucine were also capable of growth in the absence of exogenously supplied dTMP, suggesting that the plasmid born TMP1 genes were present and functional. 0.2 ml of freshly saturated cultures of both BB30-3-pTL1 and BB30-3-pRS(cen3)471 was evenly spread onto agar growth plates containing 25 ml of minimal media (2x YNBD). 200 ul of a 10 mg/ml solution of FdUMP (40mM) was placed in the wells cut in the center of the plates. After incubating the plates for 48 hours, the zone of growth and the zone

of growth inhibition were measured. The size of the zone of growth inhibition is a good indication of the FdUMP resistance of a strain. Such FdUMP well tests have been previously used to measure the relative efficiency of FdUMP uptake of the different classes of tup mutants (Bisson and Thorner 1982). Strain BB30-3-pTL1 was not at all affected by the concentration of FdUMP used, giving a complete lawn of growth all the way to the edge of the well. In contrast, BB30-3-pRS(cen3)471 gave a central zone of inhibition extending out 2.5 cm from the well. Thus it can be concluded that increased levels of thymidylate synthase resulted in increased resistance to FdUMP which suggested that the excess thymidylate synthase was functional.

DISCUSSION

SECTION 1: Factors effecting the levels of thymidylate

In this work, levels of TMP1 gene product were followed during batch culture growth and during mating \ pheromone induced G1 arrest. The major findings were: (1) levels of thymidylate synthase activity were much higher in actively proliferating cells than in quiescent cells or in α -factor induced G1 arrested cells, (2) inactivation of thymidylate synthase played a major role in determining activity levels, and (3) the mechanism responsible for inactivation of thymidylate synthase does not effect the bulk of cellular protein.

Thymidylate synthase activity is elevated in rapidly proliferating cells and reduced in stationary phase cells

Evidence has been presented to show that thymidylate synthase activity in log phase cells is at least two orders of magnitude higher than levels in stationary phase cells (fig. 1 and table 3). These results are in basic agreement with the data obtained for several higher eukaryotes which showed that thymidylate synthase levels were low in G₀ (quiescent) cells but activity

increased dramatically when growth resumed (reviewed in Prescott 1976). It was shown that inactivation of thymidylate synthase activity played a major role in the observed decrease in yeast cells (fig. 1). This is in contrast to the situation in mouse 3T6 cells, where thymidylate synthase was found to be more stable than the bulk of cellular proteins (Jehn et al. 1985).

In mammalian cells, both thymidylate synthase and thymidine kinase play important roles in the synthesis of dTMP. Thymidylate synthase is an important enzyme in de novo synthesis of dTMP while thymidine kinase plays an important role in the salvage pathway of dTMP synthesis (reviewed in Kornberg 1980). In mouse 3T6 cells, thymidine kinase was found to be unstable in vivo (Johnson et al. 1982) while thymidylate synthase was extremely stable (Navalgund et al. 1980; Jehn et al. 1985a). The loss of thymidylate synthase activity as log phase yeast cultures entered stationary phase (fig. 1) was similar to the observed loss of thymidine kinase activity in mouse 3T6 cells (Johnson et al. 1982). These results suggested that thymidine kinase and thymidylate synthase may be subjected to similar post-translational regulatory processes in the 2 different cell types.

In contrast to the high stability of the thymidylate synthase protein in mouse 3T6 cells (a half-life of around 25 hours; Navalgund et al. 1980; Jehn

et al. 1985a), thymidylate synthase has been shown to be lost in a human gastrointestinal cell line with a half life of around 6 hours (Washtien 1984). Therefore the relative degradation rates of thymidylate synthase appears to vary in different cell types.

Thymidylate synthase levels correlate with cell growth status

Many enzymatic activities have been correlated with overall growth rates. It has been shown that most enzymatic activities directly involved in DNA synthesis including thymidylate synthase, DNA polymerase, thymidine kinase, thymidylate kinase are low in quiescent cells and elevated in proliferating cells (reviewed in Prescott 1976). In yeast, Kay et al showed a close correlation between the levels of ornithine decarboxylase and growth (Kay et al. 1980). The specific activity was found to be high in growing cells and low in non-proliferating cells. Although the correlation was quite good, it was shown to be imperfect. When stationary phase cells were placed in fresh media, the beginning of DNA synthesis preceded an increase in ornithine decarboxylase activity by several hours. Furthermore growth continued when ornithine decarboxylase expression was repressed.

The results presented in this study showed that thymidylate synthase activity is an excellent indicator of

the growth status of yeast cultures. Levels were shown to be high in proliferating cells and decreased in quiescent cells eventually reaching the limits of detection (fig. 1 and table 3). In addition, thymidylate synthase levels increased within 20 minutes (earliest sample point) of resuspending stationary phase cells in fresh media (fig. 2A). The increase in thymidylate synthase preceded detectable increases in total protein content by as much as 80 minutes (fig. 2B). Furthermore the increase in activity has also been shown to precede DNA synthesis as determined by the appearance of small budded cells (fig. 2). Yeast cells which have stopped dividing in response to the addition of α -factor lost thymidylate synthase activity even though total protein continued to increase (fig. 3). Finally thymidylate synthase levels increased in response to a decrease in the cell doubling time (table 5 and see below). It would therefore appear that thymidylate synthase levels are a reliable measure of the growth status of a yeast culture. This point was further strengthened by the fact that thymidylate synthase activity was shown to be absolutely essential for the proliferation of wild type yeast cells (Bisson and Thorner 1977).

Thymidylate synthase is irreversibly inactivated by a selective mechanism

Three observations suggested thymidylate synthase was irreversibly inactivated.

(1) When stationary phase cells were stimulated to grow by resuspension in fresh media, the rapid increase in activity, which reestablished log phase levels within four hours, is prevented by cycloheximide (fig. 2A). Therefore, protein synthesis is required for the increase in activity following refeeding.

(2) Mixing extracts from stationary phase cells having low levels of activity and extracts from log phase cells having high levels of activity resulted in additive levels of activity in the resultant mixtures (table 4). If an activator or an inhibitor were involved and present in one of the extracts, mixing should not have produced additive levels of activity.

(3) The bursts in thymidylate synthase accumulation observed following refeeding stationary phase cells and following release from α -factor induced G₁ arrest, coincide with rapid increases in thymidylate synthase mRNA (E.M. Calmels, pers. comm. and see Storms et al. 1984). Proportionality between levels of thymidylate synthase mRNA and rates of accumulation of thymidylate synthase activity suggested that the reappearance of activity resulted from new synthesis.

The bulk of yeast proteins are degraded at the

rate of 0.5 to 1% per hour in log phase cultures growing on glucose (López and Gacendo 1979). The degradation rate was shown to increase 2 to 3 fold during starvation (i.e. stationary phase). During entry into stationary phase, thymidylate synthase activity was lost at a faster rate than the rate of degradation of bulk protein (fig. 1). It should be noted that the rate at which activity disappeared provided a minimum estimate of the turnover rate because it did not take into consideration the contribution of ongoing synthesis. When the TMP1 promoter directed the synthesis of a stable β -galactosidase protein (fusion 535), synthesis continued late during stationary phase (fig. 6) at a time when thymidylate synthase activity was decreasing (fig. 1). Furthermore significant levels of thymidylate synthase mRNA were shown to be present late during the growth cycle (E.M. Calmels, pers. comm.) at a time when thymidylate synthase activity was actually disappearing. It then seemed likely that synthesis was continuing, although at a reduced rate, suggesting the turnover rate was significantly greater than the rate measured.

During nitrogen starvation, the turnover of bulk yeast proteins was shown to be reduced by 60 to 75% in a pep4-3 background (Jones 1983), while the disappearance of thymidylate synthase activity was unaffected in a pep4-3 background (fig. 1). The pep4-3 mutation results

in a dramatic reduction in the activity levels of several vacuolar based hydrolases including the major proteases (Jones 1983). With similar experiments to those reported here, Sumarada and Cooper have shown that allophanate hydrolase activity disappeared during entry into stationary phase at about 3% per hour (Sumarada and Cooper 1978). The loss of allophanate hydrolase activity was thought to occur at the same rate as the rate loss of the bulk of cellular proteins (3% per hour, see Lopez and Gacendo 1979; reviewed by Jones 1983). Under the same conditions, the loss of thymidylate synthase activity occurred at a 3 fold greater rate (approximately 10% per hour, see fig. 1).

The demonstration that thymidylate synthase activity increased at a far greater rate than total protein during refeeding experiments (fig. 2) was further evidence that thymidylate synthase activity was lost during stationary phase at a far greater rate than total protein. Finally, thymidylate synthase activity decreased during α -factor induced G1 arrest (fig. 3) while the bulk of protein synthesis continued unaffected (fig. 3; Throm and Duntze 1970). Taken together these results suggested that inactivation of thymidylate synthase occurred by a selective process not effecting the bulk of yeast protein.

Thymidylate synthase levels increased in response to an

increase in the growth rate

There is an excellent negative correlation ($r = -0.998$) between the cell doubling time and thymidylate synthase levels in yeast (table 5). These results are in contrast to the results obtained by Bisson and Thorner who noted no apparent differences in thymidylate synthase levels in yeast cells grown in rich and minimal media (Bisson and Thorner 1977). Such a growth rate dependence has previously been found for certain cellular constituents such as ribosomes. In yeast, the regulated synthesis of the different ribosomal-proteins and ribosomal-RNAs has been shown to account for the growth rate dependent accumulation of ribosomes (reviewed in Warner 1982).

To explain the growth rate dependence of thymidylate synthase activity, I would like to propose the following model. Control of cell division in Saccharomyces cerevisiae occurs within the G1 portion of the cell cycle at a step defined as "start" (Hartwell 1974). Completion of the event called "start" necessitates the completion of the cell cycle. Therefore prior to the completion of "start" the cell monitors its environment to determine whether suitable conditions exist to undertake cell division. Nutritionally limited (i.e. stationary phase) and α -factor arrested cells stop cell division in late G1 just prior to the completion of start. When the

generation time of a eukaryote is increased, the percentage and the amount of time spent in the G₁/G₀ phase of the cell cycle increases, while the amount of time spent in the other cell cycle stages remains constant (reviewed in Pardee et al. 1978). Elongation of the G₁ portion of the cell cycle does not include the small portion of late G₁ which exists after "start". Therefore like S, G₂, and M, the time spent in the G₁ portion past start remains constant (reviewed in Pringle and Hartwell 1981). Thymidylate synthase synthesis occurs primarily in a short time interval just after the completion of "start" (Storms et al. 1984). In addition, there is a net loss of thymidylate synthase activity in cells arrested just prior to "start" due to entry into stationary phase or to α -factor induced G₁ arrest (fig. 3). Therefore as growth rates decrease, the period of thymidylate synthase synthesis remains constant while the period of net thymidylate synthase loss increases. This may account for the decrease in thymidylate synthase levels observed with increasingly slower growth rates.

SECTION 2: Genetic approach

Expression, in yeast, of a series of fusions involving the yeast TMP1 gene and the E. coli lacZ structural gene, was studied. The 4 fusion genes encoded fusion proteins containing a constant portion of β -galactosidase (the last 1015 amino acids of the 1023 amino acid protein) and an increasingly larger portion of the N-terminus of thymidylate synthase (13, 97, 110, or 190 amino acids).

Measurements of the steady state levels of β -galactosidase showed that activity levels increased in response to increasing amounts of TMP1 structural information in the fusion (table 5). The activity levels expressed from the largest fusion gene was at least 20 times higher than the levels expressed from the smallest fusion gene. No satisfactory explanation has yet to be found which would account for the observed differences in β -galactosidase activity obtained with the different TMP1 - lacZ fusions. All fusion genes expressed β -galactosidase activity from an identical portion of the TMP1 gene, thereby ruling out possible differences in promoter strength. It is possible that the 4 fusion genes produced identical amounts of fusion protein but the increasing amounts of thymidylate synthase at the

N-terminus renders the β -galactosidase protein more effective at cleaving the synthetic substrate ONPG. This possibility is unlikely considering the results obtained by other investigators. Fowlin and Zabin have purified different hybrid fusion proteins involving β -galactosidase and a variety of E. coli proteins (Fowler and Zabin 1983). They determined that removal of 14 N-terminal amino acids or the addition of greater than 300 amino acids to the N-terminus of β -galactosidase does not greatly alter its specific activity (U/mg of purified β -galactosidase protein).

Many groups have reported on the construction and the characterization of a series of fusion genes involving a constant portion of lacZ and increasing amounts of a yeast structural gene (see below). Emr et al. have fused variable portions of SUC2 structural gene to lacZ (Emr et al. 1984). These fusion genes encoded β -galactosidase fusion proteins with 65 to 515 SUC2 N-terminal amino acids. Antibody studies suggested that all proteins are synthesized in equal amounts. In addition, the β -galactosidase specific activity of the fusion proteins was decreased only in the 2 largest fusions containing 495 and 515 N-terminal SUC2 amino acids. Up to 400 N-terminal SUC2 amino acids were fused to β -galactosidase without affecting its specific activity. At least 4 other similar sets of fusions have been

reported in the literature. These fusions involved a constant portion of lacZ and increasing amounts of 4 different yeast genes including R-PROTEIN L3 (Moreland et al. 1985), R-PROTEIN 51A (Gritz et al. 1985) and the regulatory proteins ALPHA 2 (Hall et al. 1984), and GAL 4 (Silver et al. 1984). β -galactosidase levels of the different fusions were found to decrease or remain the same with increasingly larger portions of the N-terminal of the yeast proteins. Detrimental physiological effects of excess amounts of the larger fusion proteins, which retained some function of the native yeast protein, probably accounted for any observed decreases.

Characterization of the mechanism responsible for the in vivo loss of β -galactosidase activity

The loss of enzymatic activity has been shown occur in 2 ways. The first involves degradation of the protein and results in irreversible inactivation and loss of antigen. The second involves covalent modifications (i.e. phosphorylation) and gives rise to a reversible inactivation with no loss of antigen. Emr. et al. have constructed a series of SUC2 - lacZ fusions containing 65 to 535 N-terminal amino acids of SUC2 (Emr et al. 1984). The invertase β -galactosidase protein fusions were found to be highly stable in yeast. Furthermore, there was no apparent inactivation of β -galactosidase activity by

covalent modifications since β -galactosidase antigen levels were found to be proportional to activity levels. Results such as these suggested that there is no inherent reason for yeast cells to degrade or inactivate β -galactosidase fusion proteins.

The thymidylate synthase β -galactosidase fusion proteins containing the first 97 (fusion 757) or 110 (fusion 269) N-terminal amino acids of thymidylate synthase have been shown to be extremely unstable. β -galactosidase activity of the fusion proteins 269 and 757 decayed with a 40 minute half life after the addition of cycloheximide to a log phase culture (figs. 8 and 9). In contrast, the fusion protein containing the first 13 N-terminal amino acids of thymidylate synthase (535) was extremely stable, with no detectable loss of activity five hours after the addition of cycloheximide (fig. 8D). β -galactosidase encoded by the control fusion (760-ryp3), which contained no thymidylate synthase amino acids, was also extremely stable (fig. 8E). Therefore, the first 110 amino acids of thymidylate synthase targeted the large β -galactosidase protein (1015 amino acids) for inactivation.

The process by which β -galactosidase activity of protein fusion 269 was lost resembled proteolysis because, the loss of 269 β -galactosidase activity was completely dependent on adequate levels of energy. Inhibitors of

energy metabolism have been shown to block general protein degradation (Lopez and Gacendo 1979; reviewed in Goldberg and St. John 1976). The results presented in figure 9 showed that cyanide completely blocked the loss of β -galactosidase activity of fusion 269 which normally occurs following the addition of cycloheximide to a log phase culture. De novo protein synthesis was required to regain 269 β -galactosidase activity lost during α -factor arrest and during the transition from log phase to stationary phase. This suggested that the loss of 269 β -galactosidase activity occurred by an irreversible process. These results are consistent with the notion that β -galactosidase activity was lost because of proteolytic inactivation.

The N-terminal amino acids of thymidylate synthase were necessary for the synthesis of the different β -galactosidase fusion proteins. This is because there is no translational start site within the last 1015 amino acids of the β -galactosidase protein (portion of protein used in the fusions). In the fusions, the ATG was supplied by the TMP1 gene. Once the fusion protein has been made, the N-terminal amino acids of thymidylate synthase were not required for β -galactosidase activity since the truncated β -galactosidase protein retained full enzymatic activity (Fowler and Zabin 1983). Therefore a single proteolytic cut in the thymidylate synthase portion of

fusion protein 269 will not suffice to cause loss of β -galactosidase activity. It has been shown that an ALPHA2 - lacZ fusion was subjected to proteolysis in yeast (Johnson and Herskowitz 1985). A single proteolytic cut has been shown to occur in the ALPHA2 protein portion without effecting β -galactosidase activity. It therefore appears that the thymidylate synthase "inactivation signal" must therefore affect sequences which are removed from itself. I suggest that a thymidylate synthase "inactivation signal" can target the whole protein for irreversible inactivation.

The first 63 amino acids of ribosomal protein 51A (Gritz et al, 1985) and the first 110 amino acids of thymidylate synthase (this study) have been shown to be sufficient to target the stable β -galactosidase protein for rapid inactivation in yeast. It therefore seems that fusions between yeast genes and the E. coli lacZ structural gene will serve as powerful tools in the search for the mechanisms involved in selective protein degradation.

Two different experimental approaches can now be used to further characterize the "degradation" process. The first involves antibody studies. Antibodies directed against β -galactosidase (commercially available) can be used to follow the fate of the β -galactosidase protein during the observed losses in activity. A close parallel

between the loss of β -galactosidase activity and of β -galactosidase antigen would strongly suggest proteolytic inactivation (reviewed in Jones 1983). The second involves a search for mutants. Mutant cells that cannot "degrade" 269 β -galactosidase activity could be obtained. The selection procedure would be based on the fact that 269 β -galactosidase activity was rapidly lost during the transition from log phase growth to stationary phase (fig. 6). β -galactosidase activity would be measured in single colonies (plate assay). The presence of β -galactosidase activity in old (stationary phase) colonies will indicate a failure to recognize and or "degrade" the fusion protein (these cells may be inviable, therefore it may be necessary to search for temperature sensitive mutants). The availability of such mutants may allow the "degradation" process to be characterized. Furthermore, the isolation of such mutants would allow the cloning and characterization of genes involved in the process. Finally, experiments could be performed which would allow the determination of the minimum number of thymidylate synthase amino acid residues required for the "inactivation signal". This may be accomplished by in vitro DNA manipulations (i.e. Bal 31 generated deletions) or by in vitro or in vivo mutagenesis of the fusion gene followed by sequencing the genes producing stable hybrid proteins.

Did the instability of thymidylate synthase β -galactosidase fusion proteins mimic the instability of thymidylate synthase?

The instability of the 2 largest fusion proteins (264i and 269) mimicked the instability of thymidylate synthase when log phase yeast cultures entered stationary phase (compare fig. 1 with fig. 6), during α -factor induced G1 arrest (compare fig. 3 with fig. 7), and following S period in synchronous cultures (R.W. Ord et al., manuscript in preparation). Peculiarities arose when we compared the relative stabilities of the different fusion proteins among themselves and to thymidylate synthase itself following the addition of cycloheximide to log phase cultures. Numerous attempts were made, using cycloheximide, to determine the exact half-life of thymidylate synthase activity. At least 3 experiments showed that thymidylate synthase activity decayed with a half-life of about 60 minutes in log phase cells. In contrast, a greater number of experiments showed random fluctuations in the levels of thymidylate synthase following the addition of cycloheximide to log phase cultures. The addition of cycloheximide to the assay mixture did not effect the in vitro determination of thymidylate synthase activity which suggested that the drug did not directly effect the enzyme assay. I have yet to figure out this strange phenomenology. Cycloheximide may well be affecting the in vivo inactivation of

thymidylate synthase. To get around this possibility the half life of thymidylate synthase may best be determined by blocking protein synthesis with agents other than cycloheximide. Possibilities include the antibiotic trichodermin and temperature sensitive yeast mutants which are defective in protein synthesis at the restrictive temperature. No such problems arose when β -galactosidase activity was followed subsequent to the addition of cycloheximide to log phase cultures (fig. 8 and 9). Therefore until the exact half life of thymidylate synthase can be determined we cannot rule out the possibility that the stability of thymidylate synthase β -galactosidase hybrids is different than that of thymidylate synthase itself.

Long fusions were all unstable. However, the longest fusion (264i, containing 190 thymidylate synthase amino acids) was about twice as stable as the fusions containing 97 (fusion 757) or 110 (fusion 269) thymidylate synthase amino acids (compare fig. 8A with 8B or 8C). One possible explanation for the observed differences in stability is that the amino acids between 110 and 190 are capable of modulating or controlling the "inactivation signal" found on the first 97 N-terminal amino acids of thymidylate synthase. The construction of a hybrid protein

containing the whole thymidylate synthase protein (270 amino acids) is now in progress. Such a fusion may exactly mimic the instability of thymidylate synthase.

Inhibiting protein synthesis and then following the loss of enzymatic activity is the easiest and most convenient way of determining decay rates or half-lives of different proteins. It should be noted that the inhibition of protein synthesis has been shown to result in a 2 fold stimulation of generalized proteolysis in log phase yeast cultures (Betz 1976). A more accurate method of determining the half-life of any protein is with the use of specific antibodies. A culture is uniformly labelled with a radioactively labelled amino acid (i.e. leucine) and antibodies are used to determine the amount of radioactivity in any given protein at time intervals following a chase with excess amounts of cold leucine.

SECTION 3: Levels of thymidylate synthase in response to alterations in the dosage of the TMP1 gene

The maintenance of extra copies of most genes, in yeast, results in a gene dosage dependent increase in the gene product (as an example see Baker et al. 1984). In contrast, very few proteins have been shown to remain at constant levels in the face of an increase in gene dosage. These include genes encoding ribosomal proteins (Warner et al. 1985; Abovich et al. 1985) and histone 2A and 2B (Osley and Hereford 1981). The mechanism for dosage compensation of yeast histone gene products functions properly in the face of 1 extra copy of either Histone 2A or 2B. The introduction of either histone gene on multicopy plasmids results in cell death (L.H. Hartwell, personal communication).

Excess thymidylate synthase is non-cytotoxic

Yeast cells did not compensate for extra copies of the TMP1 gene. Levels of thymidylate synthase were elevated in a gene dosage dependent manner with a small or a large increase in TMP1 copy number (see table 6). Similarly, thymidylate synthase levels have been shown to increase by as much as fifty fold in response to a fifty fold increase in the thymidylate synthase gene copy number

in mouse 3T6 cells (Jenh et al. 1985b). The reported overproduction of thymidylate synthase in a mouse 3T6 cell line is due to gene amplification of the chromosomal thymidylate synthase gene and the overproducing cell line was selected for by its increased resistance toFdUMP. It is not known if the whole thymidylate synthase gene, or just certain parts thereof, have been amplified. The synthesis of the excess thymidylate synthase was still under cell cycle control which suggested that the amplified genes retained their regulatory sequences (Jenh et al. 1985a). On the other hand, the cell line which contained the amplified TMP1 genes maintained a considerably higher basal level of thymidylate synthase. Therefore the possibility existed that certain regulatory sequences are lacking in the amplified genes. In addition, the high copy number of TMP1 genes (about 50) may lead to the breakdown of a regulatory mechanism capable of compensating for small increases in gene dosage.

I have apparently used the whole TMP1 gene to cause a low and a high increase in the TMP1 copy number in yeast (see table 6). The results showed that the TMP1 gene contained no regulatory mechanism to compensate for the presence of extra copies. Furthermore it was shown that increased levels of thymidylate synthase did not affect the generation times (table 6) which suggested that increased activity is not detrimental to the cell.

It is surprising that yeast did not control the absolute level of thymidylate synthase since the product of its reaction, dTMP, is toxic (Tooper et al. 1981; Barclay and Little 1981). Since thymidylate synthase is not subjected to allosteric control (Kornberg 1980), the gene dosage dependent increase in thymidylate synthase activity cannot be limited by end-product inhibition.

There are at least 2 possible explanations which can adequately explain why excess thymidylate synthase activity was apparently non-toxic. It could be that a 10 fold increase in the cellular level of dTMP is not high enough to be detrimental. The second and more likely possibility is that thymidylate synthase is not the control point or the only control point for the production of dTMP. There exist several published reports supporting the second possibility. Neuhard et al. have looked at thymidylate synthase in Bacillus subtilis (Neuhard et al. 1978). They obtained a mutant which had only 8% of the wild type level of thymidylate synthase. These mutants grew normally and contained wild type levels of intracellular dTTP. In contrast, the dUMP pools were low in wild type cells and ten fold higher in the mutants. These results suggested that the availability of dUMP, and not the levels of thymidylate synthase (which are normally in excess) actually control the production of dTTP. Similar conclusions were reached by Jackson (Jackson 1978)

who looked at deoxyribonucleotide pools in a rat hepatoma cell line. He and others have suggested that dTTP allosterically inhibits the production of dUMP by feedback inhibition on ribonucleotide reductase and thereby limiting the availability of dUMP (reviewed in Kornberg 1980). Such a mechanism would require adequate levels of thymidylate synthase but it would not be effected by excessively high levels of thymidylate synthase.

Considering the above arguments, it is somewhat strange that eukaryotic cells would have evolved strong regulatory mechanisms capable of controlling the cellular levels of thymidylate synthase. Increased thymidylate synthase activity levels were shown to be confined to the late G1 and early S phases of the cell cycle in a number of different eukaryotic cell types including mouse 3T6 cells (Navalgund et al. 1980), algae (Bachmann et al. 1983), and yeast (Storms et al. 1984). In addition, post-translational inactivation of thymidylate synthase activity played a major role in governing thymidylate synthase levels in yeast. This is especially apparent in stationary phase cells (fig. 1) and in G1 arrested cells (fig. 3).

Identification of two differently sized thymidylate synthase protein?

The gene dosage dependent increase in thymidylate

synthase levels observed with the ^3H release assay was also confirmed with the $[6\text{-}^3\text{H}]\text{-FdUMP}$ technique. The Coomassie blue stained gel and the resulting autoradiograph (fig. 10) showed that a 32000 MW protein species was responsible for the bulk of $^3\text{H}\text{-FdUMP}$ binding and probably represents the native thymidylate synthase monomer since its size agreed with previously published reports (Bisson and Thorner 1981; Storms et al. 1984). In addition, the autoradiograph revealed a second $^3\text{H}\text{-FdUMP}$ band. This second band was present at a small fraction of the putative thymidylate synthase band. In fact, it was undetectable in the untransformed strain, and it was more abundant with increasing gene dosage. Furthermore since the band was larger (MW 36000) than the major species (MW 32000) it was not simply a degradation product. Ubiquitin is a 76 amino acid polypeptide which covalently attaches to proteins and targets them for degradation (reviewed in Ciechanover et al. 1984). It is possible that the minor species of $^3\text{H}\text{-FdUMP}$ represents an intermediate in the "inactivation" of thymidylate synthase (i.e. ubiquitinated thymidylate synthase).

Thymidylate synthase levels in diploid yeast cells

Diploid yeast strains containing 1 or 2 copies of the TMPl gene were constructed by mating the appropriate haploid cells. Measurements of thymidylate synthase

activity in these diploids showed that chromosomal copies of the TMP1 gene are expressed in a gene dosage dependent manner (table 7). These results are in contrast to a previously published report which observed no apparent gene dosage effect on the levels of thymidylate synthase in diploid yeast strains (Bisson and Thorner 1977). Most structural genes in diploid yeast cells (and in most other eukaryotes) show similar gene dosage dependent increases.

The results presented in table 7, showed that a TMP1/tmpl diploid grew as fast as a TMP1/TMP1 diploid even though thymidylate synthase levels were decreased by 50%. This suggested that yeast cells have at least twice as much thymidylate synthase as was actually required to maintain the growth rates observed under the conditions used here. It should be noted that the measurements of thymidylate synthase activity and growth rates were determined for growth on minimal media (2x YNBD). It would be interesting to determine if the TMP1/tmpl diploid can maintain maximal growth rates in a richer media when the generation time is considerably reduced (table 5). Many organism maintained maximal growth rates with lower than wild type levels of thymidylate synthase under growth conditions where thymidylate synthase was the sole source of dTMP (i.e. no exogenously supplied thymine). The list includes Bacillus subtilis (8% of wild type levels;

Neuhard et al. 1978), a cultured mouse cell line (about 15% of wild type levels; Ayusawa et al. 1981), cultured chinese hamster cells (50% of wild type levels; Li and Chu 1984), and E. coli (approximately 5% of wild type levels; Belfort and Pedersen-Lane 1984).

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