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**LA THÈSE A ÉTÉ
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Periodic Expression of the TMP1 Gene
of Saccharomyces cerevisiae Which
Encodes Thymidylate Synthase.

Robin Ord

A Thesis
in
the Department
of
Chemistry

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

April 1987

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ABSTRACT

Periodic Expression of the IMP1 Gene of Saccharomyces cerevisiae Which Encodes Thymidylate Synthase.

Robin Ord, Ph.D.
Concordia University, 1987

Thymidylate synthase (TS) from Saccharomyces cerevisiae was found to be expressed in a cell-cycle stage dependent (periodic) fashion. Instability of the TS enzyme contributes to a periodic fluctuation in its activity levels. Periodic fluctuations in the levels of TS mRNA suggest that the fluctuation of TS activity is determined by its periodic synthesis.

Gene fusions placing the E. coli lacZ gene encoding β -galactosidase (β gal) under the control of 5' regulatory sequences from IMP1, the yeast gene encoding TS, were constructed. The IMP1'-lacZ gene fusions gave rise to TS'- β gal protein-fusion products with β -galactosidase activities, in a periodic fashion, as follows. First, a 0.3 Kb IMP1 sequence 5' to the coding region directed high levels and periodic expression of a stable β gal activity from a IMP1'-lacZ fusion gene which contained only 13 TS codons. This fusion was therefore regulated at the level of its mRNA. Second, IMP1 sequences within the coding region conferred instability on the fusion gene product, since the longer TS'- β gal product of a fusion gene containing 110 TS codons yielded periodic peaks in activity.

Within the 0.2 Kb regulatory region and 5' to the predicted transcriptional start site, two tandemly repeated CGCG elements together

with 30 bp of intervening A-rich DNA are required for the normal levels and periodicity of TS'-Bgal activity accumulation, and for its regulation by the yeast mating type hormone, alpha-factor. This element is similar to those found 80-200 bp 5' to other cell-cycle regulated genes which are expressed at a similar time in the cell cycle.

It is further suggested that periodic fluctuations in TS mRNA translatableability may contribute to periodic changes observed in TS expression.

DEDICATION

To Hazel

for giving me the strength to start this thesis

and

to my parents

for giving me the strength to finish it.

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I would like to thank my supervisor, Dr. R.K. Storms for his patience and critical thinking, Drs. M.J. Kornblatt and M. Herrington for their advice and facilities, and the other members of my committee for their unbiased criticism. I actually enjoyed the defence.

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I owe the inhabitants of upper Overdale Avenue, as well as the families of the lab folk, a word of thanks for putting the research side of life in perspective with other realities. Finally, my two brothers, Clive and Roger have provided essential long distance support in this endeavor.

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A. INTRODUCTION

This thesis examines the cell cycle-stage-dependent regulation of yeast thymidylate synthase (TS; EC 2.1.1.45) activity. Levels of TS activity fluctuate periodically during the cell cycle (Storms et al., 1984), and the cell cycle stage dependence is regulated both at the level of its transcript and post-translationally. To study the mechanisms responsible for this "cell cycle regulation" I constructed a series of gene fusions. The fusions placed the structural portion of the E. coli lacZ gene which encodes β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) under the transcriptional and translational control signals of the yeast gene which encodes TS, IMP1.

Although the behavior of such IMP1'-lacZ translational fusions in yeast suggests that TS is regulated at many levels, this thesis focuses primarily on cell cycle regulation of the fusion transcript. Hence, before introducing IMP1, its fusion derivatives, and the strategy used to study its cell cycle regulation, I will describe several genes whose transcripts are cell cycle regulated, and examine proposed regulatory mechanisms by which periodic fluctuations in mRNA from these genes could be elicited.

A.1. Defining a Cell cycle Regulated (CCR) Gene.

The mammalian and yeast cell cycles have been studied in some detail (Figure 1.1). These studies have employed both genetic and biochemical analysis to examine the expression of cell cycle regulated (CCR) genes and their gene products. I will define such a cell cycle regulated gene as one whose transcript levels or levels of functional gene product

fluctuate in a cell cycle-dependent fashion. Cell cycle regulation of a CCR gene's transcript is assumed to influence the appearance of its gene product. Such regulation could in theory occur by periodic transcription, periodic degradation, periodic processing or periodic translation. Periodic changes in RNA levels should thus not be taken to mean periodic transcription. A common assumption, pertinent here, is that fluctuations of an mRNA species are reflected in changes in the rate of accumulation of the gene product.

Regulation of CCR genes at the level of the gene product can also be cell cycle-dependent; via processing or changes in product stability (see below). This thesis does not examine post-translational cell cycle regulation in detail; for three recent reviews of this topic see McIntosh (1986), Taylor (1986) and Greenwood et al (1986).

A.2. Review of CCR genes.

Periodic transcription of DNA into potentially translatable RNA species has only been demonstrated for the histone genes of yeast and for certain mammalian genes (see below), whereas periodic fluctuations in RNA levels have been noted for these and several other genes, including the yeast IMP1 gene studied here.

Figure 1.1 presents a genetic and physiological description of the cell cycle from yeast and mammalian cells. It should be noted that the levels of most abundant yeast transcripts do not vary during the cell cycle, although a systematic study of poly-A mRNA levels remains to be done (reviewed by Elliot and McLaughlin, 1983). In contrast, transcripts from a subset of genes, usually associated with chromosomal replication (see below), increase and decrease periodically. In this section I will critique

Figure 1.1.

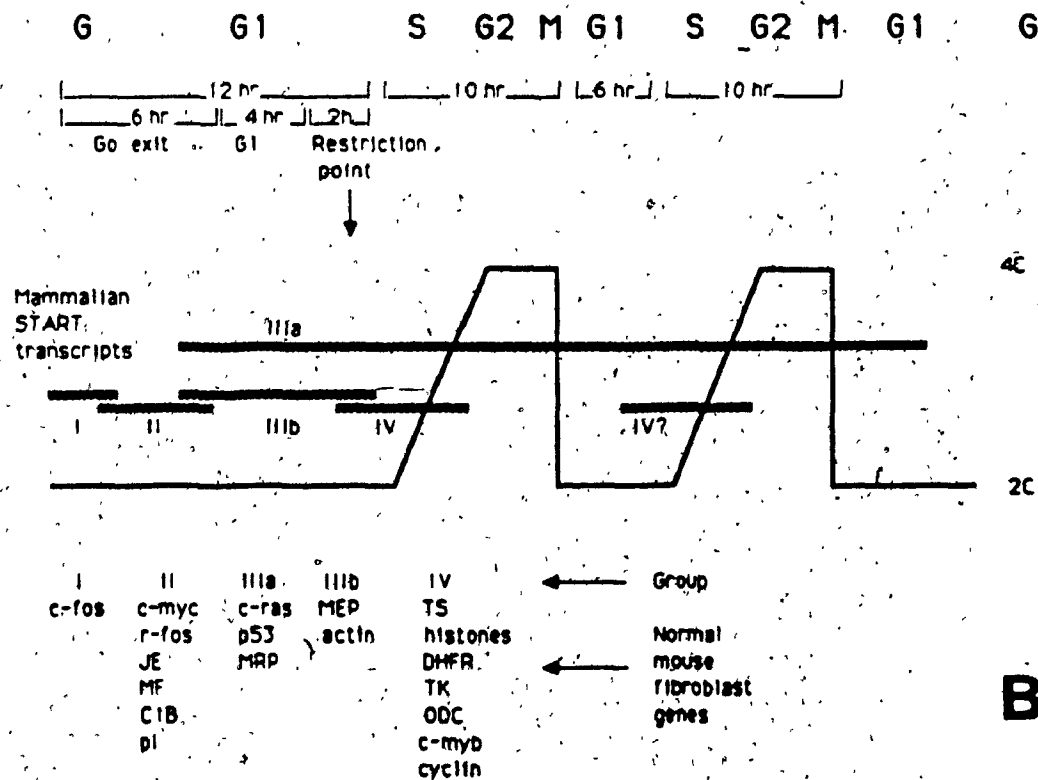
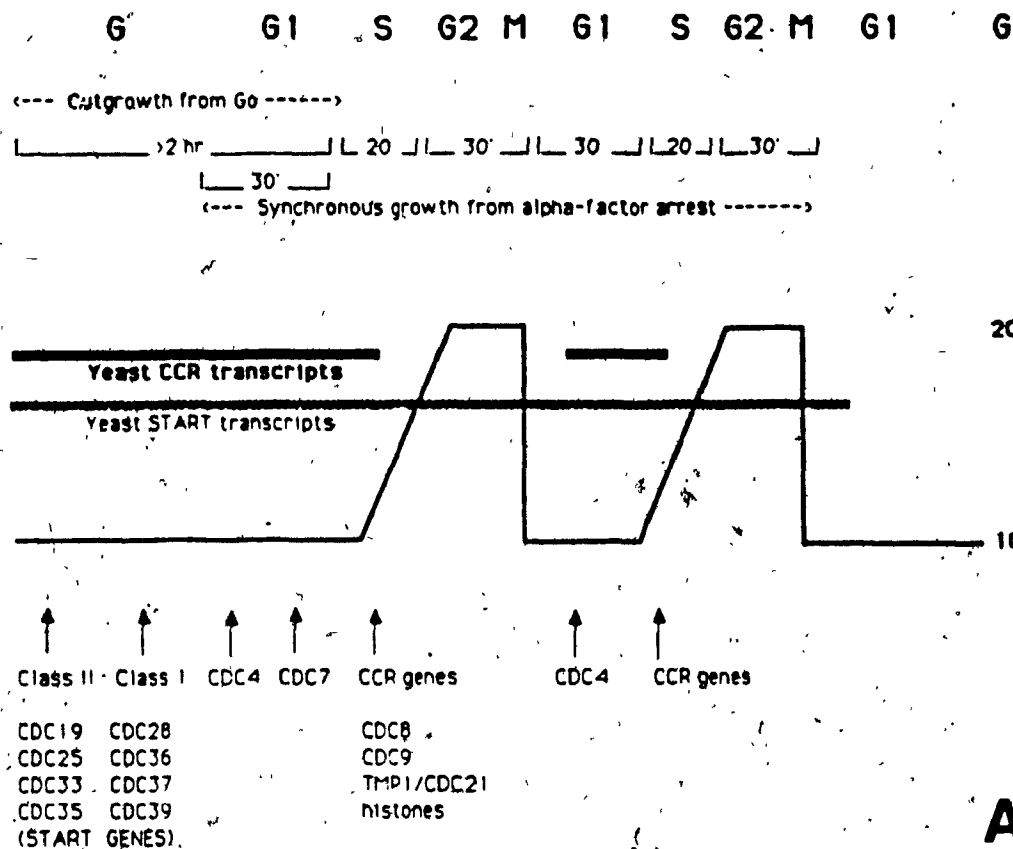
- A. "Execution points" of yeast CDC genes during two yeast cell cycles. (Hartwell, 1974). The DNA synthetic cycle is shown, cycling from 1C to 2C.

Yeast genes are conventionally designated with a three-letter symbol. Capital letters designate the dominant allele, which is usually wild-type. Mutant alleles are depicted in lower case. Mutant cdc alleles are generally conditionally lethal for growth at a "restrictive temperature", which in Hartwell's studies was 36°C. Since the appearance of cells from an arrested culture is uniform, cdc mutants are classified by their terminal phenotype. The cdc mutations thus result in a temperature sensitive phenotype. Furthermore they arrest progression through the DNA synthetic cycle at specific points. These "execution points" in the cell cycle were defined physiologically (eg. by whether DNA synthesis was initiated or completed) or cytologically (eg. by whether mitosis and cytokinesis was completed). To define more precisely the sequential order of CDC gene products within one cell cycle stage, pairwise crosses of these were made to generate cdc double mutants. The epistatic terminal phenotype conferred by one of the two cdc mutations at the non-permissive temperature indicates that its effect was determined earlier in the cell cycle (eg. Hartwell, 1974). In yeast commitment to enter the budding cycle and spindle pole body replication cycle follows the CDC28 step, commitment to nuclear DNA synthesis follows the CDC4 and CDC7 steps, and commitment to cytokinesis does not occur until after mitosis. The requirement for the expression of the CDC4 gene in each cycle subsequent to the initial cell cycle is based on the "first-cycle arrest" of the cdc4 mutant, when shifted from log phase to the restrictive condition. In contrast, most cdc mutations require multiple cell cycles to arrest under non-permissive conditions, which implies that pool sizes of required metabolites from most CDC gene products are substantial (reviewed by Carter *et al.*, 1983).

Figure 1.1

B. Timing of expression of different genes during two cell cycles, in mouse cells in culture. The DNA cycle is from 2C to 4C.

Timing of transcript appearance and/or maximum abundances in normal mouse fibroblasts (see Denhardt *et al.*, 1986, for references) stimulated to proliferate from G_0 (stationary phase) by serum addition. A time committing the mammalian cells to DNA synthesis and cytokinesis occurs just prior to S phase, and is called the restriction point. Groups III and IV require protein synthesis for their expression, whereas groups I and II are overproduced when protein synthesis is inhibited. Whether Groups I, II and IIIb change their abundance during cell cycles subsequent to the first is not known. The appearance of Group IV transcripts in subsequent cycles is inferred.



the literature concerning the regulation of several eucaryotic genes whose transcripts fluctuate periodically during the cell cycle.

A.2.1. The divergently transcribed yeast histone H2A-H2B (IRI1) locus.

The first direct demonstration of a cell cycle regulated mRNA was made by Hereford et al (1981). These authors showed that levels of histone H2A and H2B mRNA peaked early in S phase (see Figure 1.1) of the cell cycle. To demonstrate the timing of the fluctuation, the authors used yeast cultures synchronized by two different techniques. One technique involved releasing cells from a late G1 block induced by the yeast mating hormone " α -factor" (Hereford and Hartwell, 1974; described in more detail in the Materials and Methods section; the hormone's properties are reviewed by Manney and Betz, 1981). The second technique involved the centrifugal elutriation of logarithmically growing cells. This method isolates samples of cells at different stages of the cell cycle based on size (Gordon and Elliot, 1977). The relative levels of H2A and H2B mRNA in total RNA isolated from cells sampled by both techniques peaked in early S phase. The timing of S phase was monitored by DNA labelling and by correlation with bud emergence (Williamson and Scopes, 1961). It was also inferred from this study that the synthesis of H2A and H2B mRNA did not occur in the α -factor arrested cells.

The 1981 Hereford et al study also showed the following. While the levels of H2A and H2B transcripts from the IRI1 locus, and from the duplicate IRI3 locus were cell cycle regulated, RNA from genes flanking these loci did not fluctuate periodically. At least some of the RNA produced by the histone genes was translatable (using an in vitro "wheat-germ" translation assay; Roberts and Patterson, 1973). Since

this in vitro translation showed that translatable mRNA encoding each of the four histones was greatest in early S phase cells, the results suggested that all yeast histone genes were regulated like H2A and H2B.

H2A and H2B transcripts are both transcriptionally and post-transcriptionally regulated (Osley and Hereford, 1981). Two criteria suggest post-transcriptional control. First, the turnover rate of histone RNA in a strain containing two copies of the IRT1 locus is twice that of the normal, single copy-containing parent strain. Second, the half-life of H2A and H2B RNA during S phase at the permissive temperature is normally 15 minutes, while in a cdc8 temperature-sensitive mutant incubated at the restrictive temperature, the RNA half-life is only 3 minutes. The cdc8 mutation (Figure 1.1; see below) causes the DNA synthetic cycle to terminate prior to completion of chromosome replication (reviewed by Hartwell, 1974). The authors proposed that this decrease indicated that histone RNA was stabilized by ongoing DNA replication, although its rate of synthesis was not necessarily increased.

Hereford et al (1982) monitored histone RNA synthesis and histone RNA stability during the cell cycle of wild type cells, and of cdc7 and cdc8 mutant cells. Here, the cells were first synchronized in late G1 by treatment with α -factor at 23°C, then released from the α -factor block and incubated at 36°C. At 36°C the wild type cells progress through the cell cycle normally while the temperature-sensitive cdc7 and cdc8 strains are arrested. The temperature sensitive lesion results in these strains arresting their progress through the DNA synthetic cycle at the G1/S boundary, and during S phase, respectively (Figure 1.1).

In the wild type strain, transcription of histone RNA, and accumulation of histone RNA, were normal at 36°C. Transcription peaked

prior to S phase, based on the timing of maximum incorporation of radioactive label. In contrast, histone RNA levels detected by a different technique ("Northern" analysis; see below) peaked 20 minutes later in mid-S phase. Maximum incorporation of radioactive label into H2A/B RNA occurred 10 minutes prior to the time when histone mRNA levels were accumulating at the maximum rate. Since the maximum rate of histone RNA accumulation did not coincide with the time when histone RNA synthesis is maximal, but occurred 10 minutes later, it was suggested that histone RNA stability changed as cells progressed through the cell cycle.

These results were in agreement with the behavior of histone RNA in the cdc8 mutant described above, and suggested that histone RNA levels were not only regulated at the level of transcription, but were also regulated at the level of the RNA half-life. However, the latter results should be interpreted cautiously, because the duration of the peaks of RNA levels and of RNA synthesis were only 20 minutes, and the culture was only sampled every 10 minutes.

In cdc7 cells which were released from α -factor arrest at 37°C, levels of histone RNA increased to reach a constant level after 40 minutes. Similarly, synthesis of histone RNA increased to reach a constant rate after 25 minutes. Therefore transcription continues in cdc7 cells arrested at the restrictive temperature.

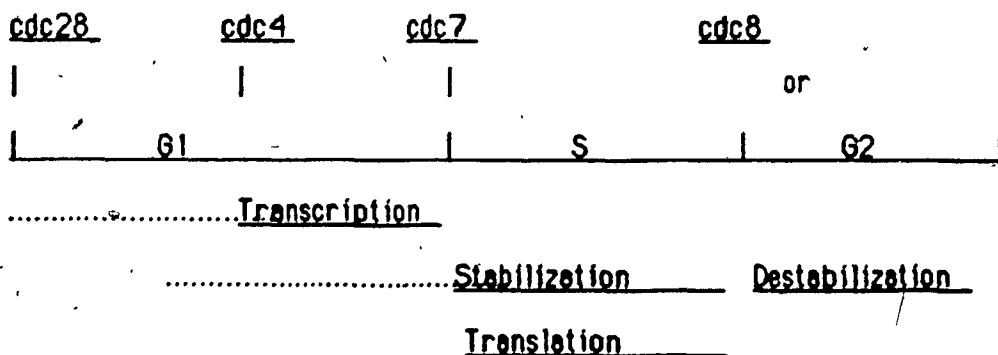
Finally, if the cell cycle was arrested during DNA synthesis at the cdc8 block, the timing of the peak of histone RNA synthesis was normal, but the peak in histone RNA levels occurred earlier than in cultures of wild-type cells. In addition, the rate of RNA accumulation was maximum when the rate of synthesis was maximum, suggesting that changes in RNA stability were no longer contributing to the profile observed.

The authors proposed that transcription could initiate earlier in the cell cycle than was suggested by Northern analysis. In this regard, White *et al* (1986) found that mRNA from H2A did not accumulate in a cdc4 mutant strain held at the restrictive temperature (see Figure 1.1). The cdc4 mutants are unable to initiate spindle pole body separation late in G1, and cannot enter S phase at 37°C. As a result, the DNA synthetic cycle is blocked in late G1 earlier than the block imposed by the cdc7 mutation, but after the α -factor arrest stage (reviewed by Carter *et al*, 1983).

In contrast to the lack of histone RNA synthesis, mRNA levels from the yeast ligase (CDC9), dTMP kinase (CDC8) and dTMP synthase (CDC21 or IMP1) genes showed a near-normal fluctuation over one cell cycle in cdc4 mutant cells held at 37°C. The simplest explanation for these data is that CDC21, CDC8 and CDC9 are transcribed earlier in the cell cycle than histone genes, and that traversing the CDC4 step is required for the transcription of the histone genes.

A model for cell cycle regulation of IRT transcripts, adapted from Hereford *et al* (1982), is given below, taking into account the data of White *et al* (1986).

H2A/B transcript regulation



Note that although histone RNA fails to accumulate prior to the CDC4 step, the possibility that transcription of unstable histone RNA is initiated earlier (shown by the dotted line) cannot be excluded, because RNA levels were followed but not RNA synthesis. Likewise, histone RNA may also be stabilized prior to the CDC7 step.

Interestingly, the 3' ends of both H2B genes contain putative ARS (autonomously replicating sequence) elements (cited in Osley and Hereford, 1982). ARS elements are capable of functioning as replication origins in yeast. Osley and Hereford (1982) proposed that periodic transcription of H2A and H2B was controlled by DNA replication origins. Plasmids containing the implicated H2B 3' ARS sequences, and the E. coli lacZ gene fused in frame (see section A.6.) to the 5' sequences of the IRT1 H2A gene, were constructed and transformed into yeast. In synchronized cells which contained this plasmid integrated at the non-cell-cycle regulated leu2 locus, the plasmid-encoded β -galactosidase gene product expressed its activity as a step. In contrast, a similar leu2-integrated plasmid deleted for the putative ARS sequences at the 3' end of H2B yielded lower β -galactosidase activities, which increased in a linear fashion during the cell cycle. At first sight, this profile was symptomatic of non-periodic expression (Mitchison, 1971). The β -galactose activity profile of the same ARS-less plasmid integrated at IRT1 instead of at leu2 was steplike, which further supported the authors contentions that the IRT1 ARS was required to promote periodic behavior. Finally, the ARS-deleted plasmid produced 3-fold less β gal activity in yeast, and transformed cells with only 1% the efficiency of the ARS-containing plasmid.

However, in this paper Osley and Hereford (1982) presented β gal.

activities for synchronized cells as "activities per cell" (ibid, their Figure 3). Whereas a linear increase in enzyme activity per ml with time is a reliable indicator of non-periodic expression in yeast (Mitchison, 1971), a linear increase in enzyme activity per cell could only be produced if activity increased in direct proportion to the increase in cell number. Since cell number increases in a stepwise fashion in synchronous cultures (see Discussion and Figure 41, this thesis), a linear increase in activity per cell could only occur if expression was periodic, or if the experiment had been carried out for less than one generation. "Figure 3", which is critical to the authors' argument, must therefore be discounted.

Perhaps because of these difficulties, these authors recently refuted their earlier hypothesis that periodic transcription of histone mRNA was controlled by 3' flanking DNA replication origins (Osley et al., 1986). This was done by demonstrating that the RNA made from the same H2A-lacZ fusions used by Osley and Hereford (1982) fluctuated in a periodic fashion, even when the ARS was deleted and the plasmid was integrated at leu2. The timing of these periodic fluctuations in fusion RNA was very similar to the timing of the fluctuations in H2A/B mRNA from the same samples. The RNA levels from the fusion lacking the ARS were, however, considerably lower than the same plasmid with the ARS. This suggests either that the 3' end of the H2B gene is a 5' transcriptional enhancer of the H2A gene, or perhaps, that the ARS-containing plasmid is present in multiple copies.

More importantly, the 1986 Osley et al. study claimed to have localized the sequences which were necessary and sufficient to direct the periodic transcription of H2A and H2B. The deletion of DNA sequences in the 5' regulatory region situated between the divergently transcribed H2A and H2B genes of IRT1 resulted in altered levels of H2A'- (and H2B'-) -'lacZ

fusion RNA. In particular, the deletion of a 54 bp region including the dyad sequence 5'-CGTAACTATGGTTAGACG resulted in enhanced levels of H2A'-lacZ and H2B RNA. Furthermore, RNA levels no longer fluctuated periodically. The 54 bp sequence was also required to reduce H2A'-lacZ transcript levels during α -factor arrest. Therefore this element may be responsible for the reduction of histone RNA expression seen during cell cycle stages other than when the histone genes are normally expressed. This dyad has been called a CCR element by Hereford and Osley (ibid).

Three copies of a second, 16 bp sequence, the "16-mer" (5'-GCGAAAAANTNPuGAAC), are also found in the region between the H2A and H2B genes. Part of this element resembles the CACGAAAA consensus sequence described by Nasmyth and co-workers for the HQ gene (described in detail below). If all three copies of this 16 bp TRI element were deleted, RNA levels from both divergently transcribed genes were drastically reduced. Therefore this sequence has a positive effect and is required for normal H2A'-lacZ transcript levels.

Both regulatory sequences were tested separately for their ability to cause periodic fluctuations in transcript levels (Osley et al, 1986). In one experiment, three copies of the 16-mer were substituted for the UAS ("upstream activating sequences"; UAS elements have a number of features similar to transcriptional enhancers) of a second yeast gene, CYC1, whose 5' regulatory region had also been fused to lacZ (Guarente and Plashne, 1981). Cells were once again synchronized using the α -factor arrest-release method. The substitution of the 16mers for the CYC1 UAS results in a peak in CYC1'-lacZ transcript levels during the first synchronous generation, at the same time in the cell cycle that H2B RNA levels peak (ibid, their Figure 5). In the presence of the CYC1 UAS, the CYC1'-lacZ fusion RNA levels did not fluctuate periodically (but see

Results section below). This result was interpreted to mean that CYC1'-lacZ RNA fluctuated periodically only in the presence of the IRT1 16-mer. However, because CYC1'-lacZ RNA was not monitored for more than one generation in this experiment, one cannot exclude the possibility that the 16-mer may act to transiently enhance histone message levels, as a function of release from the arrested state.

Next, a synthetic oligomer containing the CCR dyad was inserted 3' to the CYC1 UAS, upstream of the CYC1'-lacZ fusion. The CYC1-directed transcript from this construction gave rise to periodic fluctuations in CYC1'-lacZ transcript levels (ibid their Figure 5). This result was very similar to that obtained when the entire 60 bp CCR was inserted upstream of the CYC1 UAS, but was different from the expression seen when the 16-mer was used. Three peaks of CYC1'-lacZ RNA occurred over the same period as two H2A RNA peaks. Therefore the CCR-regulated transcript, unlike the histone RNA, seemed to be peaking more than once per generation. This result may mean that the two regulatory elements cannot be uncoupled without altering periodic expression.

The CCR affects CYC1'-lacZ transcript levels regardless of its location upstream or downstream of the CYC1 UAS. However, the full CCR (not just the dyad), when placed between the CYC1 UAS and the putative "TATA" transcription initiation region, yielded levels of CYC1'-lacZ RNA which were too low to analyse. This observation lends credence to the negative regulatory role postulated for the CCR element.

The authors comment that a third element may be required for wild-type regulation, since a non-periodic RNA profile was observed from both divergently transcribed genes when only the CCR situated between them was deleted from an H2A'-lacZ fusion (ibid their Figure 4B). That is, the "16mer" UASs, two of which were still present in these

constructs, should still have produced a periodic peak of RNA. However, the high levels of H2A'-lacZ RNA in this experiment, presumably due to the absent CCR element required for down regulation, may have masked the effect of the 16mer. Therefore, instead of an additional element being required, it is possible that the two elements act together, the 16-mers to enhance message levels after the cells were released from α -factor, and the CCR element to reduce mRNA levels at cell cycle stages other than S phase.

In summary, the data from Hereford's group indicate that periodic expression of histone transcripts is mediated by two types of DNA sequences located 5' to the structural genes, but that other elements (possibly an ARS) located 3' to the H2B sequences (and therefore 5' to H2A) are required to enhance transcription of H2A. (The authors do not state whether this ARS is also required for high levels of H2B RNA). It was further inferred that a variable RNA half-life plays a role in determining the periodic RNA profile.

A.2.2. The yeast HQ gene.

The yeast HQ gene which is responsible for initiating the switching of mating type in yeast is also periodically expressed (Nasmyth, 1982, 1983; Abraham et al, 1984).

Haploid yeast cells exist in two different mating types, called a and α , respectively. Mating type is determined by which allele, MAT_a or MAT _{α} , is present at the MAT locus located near the middle of chromosome III. Two silent (inactive) copies of MAT_a or MAT _{α} , called HML_a and HMR _{α} respectively, are distantly located at opposite ends of the same chromosome III. In haploid yeast which carry the wild-type allele of the HQ gene, mating type switching occurs when one of the silent copies

replaces the opposite mating type allele at the the MAI locus between them. The result is to generate an a cell from an α cell, or vice versa.

The switching process is apparently initiated by a double strand break (Astell *et al.*, 1981) caused by a HO-encoded endonuclease (Kostriken *et al.*, 1983). The cleavage site is at one end of the MAI locus in a region called the "Y cassette" (see Astell *et al.*, 1981). The HO cleavage site (~) in both mating type alleles is found within the sequence

5'-CTTPyG ~ CGCAACAGTA T/AA ~ (Nasmyth *et al.*, 1981).

The regulation of HO expression is complex. First, only mother cells (cells which have produced at least one bud) express HO mRNA and gene product. Second, HO is expressed in haploid cells but not in a/a diploids. Third, the expression of HO mRNA is cell cycle stage dependent. Since this thesis deals with cell cycle dependent expression my discussion of HO regulation will be confined to the HO regulatory elements which regulate its cell-cycle stage dependent expression.

Nasmyth (1983) first demonstrated that HO was periodically expressed by following HO RNA levels and HO endonuclease activity in partially synchronized cells. These synchronous populations were obtained by refeeding stationary (G_0) phase cells with fresh medium. Both HO-specific mRNA (observed as an S1-protected RNA banding at the correct size on a polyacrilamide gel; Berk and Sharp, 1978) and endonuclease activity appeared in late G_1 . That is, their appearance preceded the onset of budding which marks the beginning of S phase.

In order to further localize the timing of HO expression in the cell cycle following refeeding, Nasmyth (1983) released a series of cdc mutants from G_0 arrest using the same procedure described above. The cdc28, cdc4, cdc15 and cdc31 mutations each result in a temperature sensitive phenotype. Furthermore they arrest progression through the

DNA synthetic cycle at specific points (see Figure 1.1).

The cdc28-4 mutation causes cells to arrest at a stage in late G₁ known as START (see also section A.3.), which is the same stage at which cells are blocked by α -factor hormone (Hartford and Hartwell, 1974). Both HQ RNA and nuclease failed to appear when the cdc28-4 culture was released from G₀ arrest at the restrictive temperature. From this experiment it can be concluded that HQ is expressed late in G₁ after START. This is similar to the timing of histone gene expression (section A.2.1.).

The cdc4 mutation prevents the onset of chromosomal replication, but not the budding cycle, and the CDC4 step appears to follow sequentially the CDC28 step (ibid). When G₀ cdc4 cells are inoculated into fresh medium at the restrictive temperature, HQ RNA and gene product appear. Therefore HQ expression is detectable prior to the CDC4 execution point (Figure 1.1; Nasmyth 1983), in contrast to histone RNA.

The cdc15 mutant is defective in nuclear division (mitosis) and so can pass one, but not two, "START" or cdc28-4 points (Figure 1.1) when placed at the restrictive temperature. Results for HQ expression similar to those from the cdc4 mutant were obtained with the cdc15 mutant. Together, these results indicate that passage through START is necessary and sufficient for HQ RNA synthesis (ibid).

Strangely, the endonuclease activity peaked and disappeared in cdc4 and cdc15 mutants, whereas endonuclease from wild type cells was present continuously, when cells were released from G₀ arrest at 37°C. In contrast, the mRNA from all three strains peaked shortly after inoculation. The latter result suggests that mutant cells may accumulate at cell cycle stages when enzyme activity is lost.

Both cdc4 and cdc15 affect the mitotic spindle apparatus (reviewed

by Pringle and Hartwell, 1981). Because of this, it is possible that the stage of arrest is identical in both strains, namely at START, the onset of spindle pole body (SPB) replication in the subsequent generation. Pringle and Hartwell (ibid) proposed that SPB duplication was the START event which triggered a cell cycle (see Discussion). However, SPB duplication was not required for the second generation expression of HQ mRNA. A cdc31 mutant, which fails to replicate its SPB at the end of the first cell cycle, and so arrests in the second cycle at the restrictive temperature, still showed a second-cycle peak of HQ RNA. This result could also be interpreted to mean that, in generations subsequent to the first cell cycle, START may become associated with an event other than SPB duplication (such as the completion of mitosis; see below).

Together these results showed that HQ expression is START-dependent, and begins in late G1 prior to the CDC4 execution point.

The regulatory elements responsible for the regulation of HQ appear to be contained 5' to the gene. Although ARS consensus sequences (AAA T_A TATAAA) were detected within or 3' to the HQ gene (Russell et al. 1986), as was the case for TRP1, Nasmyth (1985a,b) has defined several different elements, all 5' to the gene, which appear to account for its regulation (see Figure 1.2. below).

Based on deletion analyses, a region (URS1) more than 1000 bp 5' to the coding region was necessary for the expression of normal levels of HQ RNA. Removal of this far-upstream region (-1000 to -1362), or insertion of the entire TRP1 gene (EcoRI - BglII fragment) at one of two sites located at -1050 and -690 respectively (numbers are relative to the first base of coding DNA) drastically reduced levels of HQ mRNA. The results with the TRP1 insertions suggest that URS1 is cis - acting.

The 140 bp immediately 5' to the coding region (Nasmyth, 1985a) was

also required for production of the correct HQ mRNA. The transcript 5' end was mapped to roughly -50, and a TATA-like sequence appears at -120 (Russell *et al.*, 1986). The proximity of TATA sequences in DNA to 5' mRNA ends has implicated them in transcription initiation (references in McNeil and Smith, 1986). A deletion (-61 to -106) between the putative TATA sequence and mRNA start sites virtually eliminated all RNA species, except a high molecular weight band.

Interestingly, deletions of more of this region; from -61 to -177 including the TATA box, caused a less severe reduction in total HQ RNA than did the smaller -61 to -106 deletion. However, the RNAs produced were shorter than HQ mRNA. In contrast, deletion of almost the same amount of 5' DNA including the putative mRNA start site itself (-21 to -54) did not significantly alter HQ mRNA levels, and only slightly altered its size. Since the RNAs produced from these different constructions, have altered 5' regions, the reductions in the HQ RNA levels from the -61 to -106 deletion might be due to changes in transcript half-life rather than to altered transcription.

A third region, URS2, was localized between -901 and -145 (Nasmyth, 1985b). Distributed throughout this region were ten related sequences, the consensus of which was CACGAAAA. Interestingly, this CACGA₄ sequence is similar to the "16mer" described by Osley *et al.* (1986; see Section A.2.1.). Nasmyth claimed that his data showed that the CACGA₄ consensus confers cell cycle "START-dependent" transcription on the HQ gene.

This implied regulatory role of URS2 was based on two observations. First, wild type HQ RNA did not appear until 60 minutes following refeeding, and then peaked and disappeared. In contrast, cells containing an altered HQ gene which lacked most or all of URS2 (ibid, deletion

229.102) was continuously present at levels equivalent to the peak levels of wild-type HQ RNA, at all times during the cell cycle except the time-zero sample. Second, HQ mRNA from the URS2-deleted gene was continuously produced, even at time-zero, in stationary phase cdc28-4 or cdc15 cells reled at the restrictive temperature. It should be recalled that these mutants arrest the DNA synthetic cycle at stages when HQ RNA is not normally expressed.

These observations indicate that the deletion of URS2, which contains all of the postulated CACGAAAA regulatory sequences, eliminates START-(CDC28) dependent regulation. Furthermore, the constitutive expression of the URS2-deleted HQ gene suggests that URS2 contains a negatively-acting component.

A fourth type of regulation was evidenced by a reduction of HQ RNA levels in wild type cells, in the presence of the α -factor mating hormone, which, like the cdc28-4 mutation, arrests cells at START. Deletions of URS2 resulted in the appearance of HQ RNA of the appropriate size whose levels were not reduced by α -factor. URS2 is therefore also associated with the reduction in RNA levels caused by α -factor. Since both the cdc28-4 mutation and α -factor arrest cells prior to the START step, URS2 appears to be involved in curtailing RNA levels in G₁ prior to completion of START.

When three to five copies of the CACGAAAA sequence were inserted in place of the entire URS2, START dependent regulation of HQ mRNA in cells outgrown from G₀ was restored. More importantly, during synchronous growth following release from α -factor arrest, HQ mRNA from a strain which contained five copies of the CACGAAAA sequence was transcribed in a manner very similar to the HQ gene. The appearance of HQ mRNA occurred slightly earlier than histone mRNA in both cases. In the absence

of the CACGAAAA sequences the transcript levels were somewhat (less than two-fold) reduced, and did not show an increase until the second generation. From this second cycle increase Nasmyth concluded that: "URS2 deletions are still regulated by cell cycle events that are close in time to START".

Nasmyth interpreted his results to mean that HQ was periodically transcribed under the regulation of URS2. One problem with this kind of interpretation is intrinsic to the methods presently available for the identification of RNA species in general. The nuclease (S1) mapping technique (Berk and Sharp, 1979) used by Nasmyth or by Hereford *et al* for quantitating RNA levels cannot distinguish transcription start sites from processing sites, nor identify the correct transcript if multiple transcripts exist from the same region. Furthermore, if the 5' regulatory region were extensive as may be the case for HQ, then a short probe such as that used by Nasmyth (1983; 1985a,b) may be unable to detect or identify upstream initiation sites.

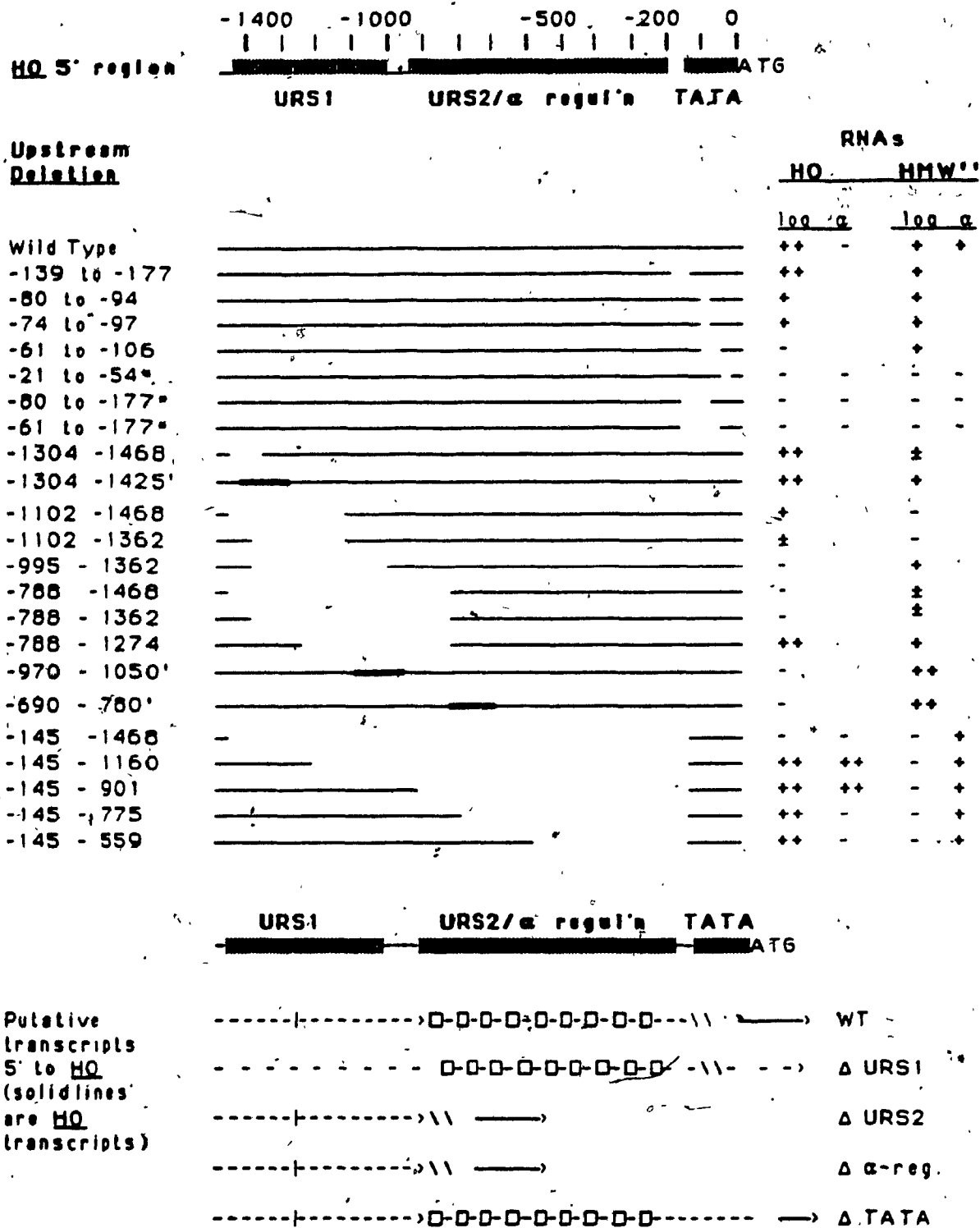
The aforementioned problems appear in the results of Nasmyth (1985a,b), where HQ RNA was detected by S1 mapping, using a 0.87 Kb probe which protected transcripts extending upstream of the translation start as far as -163. A band appeared at "high MW" (0.87 Kb) in his autoradiograms whose presence was explained as "undigested probe", but which the author conceded could be full-length protection of the probe by a transcript initiating much further upstream. Because the "high MW band" was present in α -factor arrested cells, but not in log phase cells, it seems unlikely that the band results from undigested probe DNA. The high MW band was also seen when the entire 5' regulatory region from -145 to -1468 is deleted, a situation where HQ mRNA of the predicted size is virtually undetectable (ibid their Figure 3).

An interesting alternative interpretation to the model of periodic transcription of HQ, is that HQ RNA is constitutively transcribed from a region much further upstream than Nasmyth believes. This would result in a large primary transcript (ie. the one which predominates in α -factor arrested cells), which could be responsible for the full length protection of the probe DNA. The implication is that the smaller, cell cycle regulated mRNA species could arise from the larger precursor by a processing event near a position corresponding to -50, the location of the 5' mRNA end. HQ RNA levels are altered by deletions in the -61 to -106 region (ibid). Hence this region could be where any upstream transcript is processed to produce the mature mRNA. No data presented to date eliminates either transcription or processing models for the periodic expression of HQ RNA.

As was the case with histone RNA, changes in transcript stability, rather than changes in the rate of transcription, may explain changes in the levels of HQ RNA in the different deletion mutants. However, the rate of HQ transcription rates was not measured and so, unlike the histone studies, data regarding mRNA stabilities is not yet available.

The regulation of the yeast HQ RNA by upstream sequences is summarized on the following page (Figure 1.2). Shown are the 5' regulatory elements and below these are given the various deletions which gave rise to the RNA phenotypes observed (HMW RNA is the putative upstream transcript). It can be seen from this summary that START regulation and down regulation caused by α -factor arrest may be caused by the same region, as was the case for IRI1 (Osley et al, 1986). It should also be recalled that the low HQ RNA levels observed early during θ_0 outgrowth were not observed when URS2 was deleted (Nasmyth, 1985a,b). Nasmyth (1985b) therefore concluded that the

Figure 1.2. HO RNA Regulation (adapted from Nasmyth, 1985a)



* RNA levels were at least as high as wildtype, but 5' RNA ends had altered. Furthermore, when α -factor arrested cells were tested, the sites of mRNA ends was also different from wild-type, as if α -factor influenced 5' RNA ends.

' Sites of TRPI insertions are boxed.

" HMW: high molecular weight RNAs having full length (S1) protection.

stage in the cell cycle dictated the relative levels of HQ RNA, in agreement with Osley *et al.*

Other similarities in the results of Nasmyth versus those of Hereford and Osley include the following. First, the removal of the IRI1 CCR region or of the HQ URS2 results in constitutive levels of RNA in log phase cells. Both of these regions appear to contain a negative element acting to reduce mRNA levels prior to START, and a positive element whose presence is required for the elevation in mRNA levels after passing START. Second, both HQ and H2A appear to require sequences located more than 1Kb 5' of the putative mRNA 5' ends in order to express normal RNA levels (the URS1 of HQ and the ARS of IRI1). Third, both genes contain positive elements less than 20 bp in length whose presence more than 200 bp upstream of the coding region was required for START-dependent appearance of the respective RNA.

A.2.3. The yeast CDC8 (dTMP kinase), CDC9 (DNA ligase) and CDC21/TMP1 (thymidylate synthase) gene battery.

Three other yeast genes, CDC8, CDC9 and CDC21 are known to be cell cycle regulated. Like the histone and HQ genes, these are directly associated with DNA metabolism. None have been characterized to the extent of the IRI or HQ gene, but each has been sequenced and studies of their RNA have been initiated.

CDC9 has been sequenced by Barker *et al.* (1985), and its RNA was shown to be periodically expressed by Peterson *et al.* (1985) and White *et al.* (1986). CDC21 or TMP1 was sequenced by Taylor (1986), and was shown to be periodically expressed by Storms *et al.* (1984). Birkenmeyer *et al.* (1984) sequenced the CDC8 gene. White *et al.* (1986) showed that the CDC8 gene was periodically expressed. Using Northern analyses,

White *et al* (1986) also found that transcripts from these three genes are elevated at the same time in the cell cycle. The RNA levels from these genes peak earlier in the cell cycle than do histone RNA levels. The RNAs accumulated in the presence of the cdc4 block, suggesting that their transcription was initiated in G1 prior to this cell cycle step (Figure 1.1).

As discussed in detail later (Section D.2.), these three CDC genes share a repetitive DNA element located 100-200 bp 5' to the structural gene, the consensus of which is:

5'-C8C8-----A-rich-----C8C8

The intervening A-rich region is 4-30 bp. This sequence may be a CCR element, since I have only found this sequence 5' to CCR genes, to date.

Based on the similar timing of peaks in the RNA levels during the cell cycle, White *et al* have already proposed that these three genes are members of a cell cycle regulated gene "battery" (Britten and Davidson, 1969). To date, no studies have reported the location of regulatory sequences for these genes, although both IMP1 (Taylor, 1986) and CDC9 (Barker *et al*, 1985) have mRNA ends mapping less than 60 bp from their predicted translation start sites and downstream of potential "TATA" sequences (see Figure 1.3, for example). As with histone RNA, CDC9 RNA remains elevated during cdc7 - induced arrest (Peterson *et al*, 1985). This suggests that cells must traverse the CDC7 step before transcription of the CDC8, CDC9, CDC21, H2A and H2B genes can be turned down.

A.2.4. Mammalian cell cycle genes.

The mammalian tissue culture system has also been used for studies of transcript regulation during the cell cycle (Denhardt *et al.*, 1986). Here, too, the cell cycle is beginning to be elucidated using cell division cycle mutants and onc genes (*ibid.*, see also Sheinin, 1985, 1986 and reviews by Pardee *et al.*, 1978; Marcus, 1985).

The RNA product of the gene encoding the 70 kD human "heat shock protein", HSP70, represents a mammalian RNA species which fluctuates periodically. The highly conserved HSPs (reviewed by Lindquist, 1986) are induced when the cell is stressed by a number of agents, including high temperature shocks. The human HSP70 has 50% homology to the *E. coli* dnaK gene product, which is an abundant autophosphorylating protein with weak ATPase activity. The dnaK gene is an essential gene for *E. coli*, in that it cannot be deleted, and is also required for replication of phage lambda (*ibid.*). In yeast, there are two HSP70 proteins, which accumulate relative to other proteins as cells enter stationary phase. Like the analogous *E. coli* dnaK gene, the yeast HSP70 gene defined by mutant YG104, is essential for growth (*ibid.*).

The abundance of the RNA encoding a human HSP70 protein increases 20-fold during the cell cycle, reaching a peak during the post-S phase period (Kao *et al.*, 1986). Periodic expression of human HSP70 RNA is observed from HeLa cells and from Adenovirus-transformed 293 cells released from a thymidine aphidicolin cell cycle block (*ibid.*). This block synchronizes cells at the G1/S boundary. However, the peak of HSP70 RNA occurs after DNA synthesis, which probably means that the HSP70 gene is not related to the CCR gene battery of yeast (section A.2.3), whose transcripts peak near the beginning of S phase.

The levels of several transcripts from the human adenovirus linear

genome were also found to fluctuate in a cell cycle-dependent fashion in chronically infected cells (Kao *et al.*, 1986). Like the SV40 and Herpes viruses (reviewed by Tooze, 1980), adenovirus contains a "master" gene, E1A, whose protein product regulates expression of all of the other genes encoded by the virus. The 5' sequences of the E1A gene serve as a transcriptional enhancer for all other early viral genes (Hearing and Shenk, 1986). In addition, the E1A product can elevate cellular HSP70 and β -tubulin RNA levels (Kao and Nevins, 1982). These data suggest that the E1A gene product is capable of controlling periodically expressed genes, such as the HSP70 gene in the cell's genome. However, since evidence for cell cycle regulation of β -tubulin RNA levels is lacking, the action of E1A on transcription of these genes may be an indirect result caused by stimulation of proliferation (see section A.3.).

This ambiguity was partially addressed by studies of the regulation of RNA levels from the adenovirus E1A and E1B genes (Kao *et al.*, 1986). Mouse 293 cells chronically infected with adenovirus accumulate RNA from these viral "early" genes in a manner consistent with cell cycle dependent regulation. That is, levels of E1A and E1B RNA fluctuated during a round of cell division in synchronized 293 cells. Accumulated levels of both RNAs were low at the G1/S boundary, peaked in early S phase 6-9 hours after release, and fell again to low levels by 18 hours. The peak in both E1A and E1B RNA levels occurred 3-6 hours before the HSP70 RNA peak.

The rate at which the E1A, E1B and HSP70 genes were transcribed during the cell cycle was also measured as described above for yeast histone RNA. Whereas incorporation into HSP70-hybridizing RNA was not observed until 4 hours into S phase, incorporation into E1A RNA was maximal at the G1/S boundary and declined through the rest of the cycle.

Incorporation into E1B RNA was constant throughout the cell cycle. The results imply - and the authors suggest - that the periodic fluctuations of E1A is both transcriptionally and post-transcriptional regulated, while the periodic expression of E1B is post-transcriptionally regulated.

The other important conclusion is that E1A and E1B transcription begins before the G1/S boundary, whereas HSP70 transcription only occurs when the cells have entered S phase. This conclusion is based on the timing of the cell cycle block used to synchronize the 293 cells studied here. Since the cells were arrested at the G1/S boundary prior to these experiments, RNA levels which were high at the beginning of the experiment are assumed to have been caused by increased synthesis (as with yeast histone RNA during a cdc7 cell cycle block; section A.2.1.), and/or by stabilization, in the arrested cells. In either case transcription takes place at the block, and may have been initiated much earlier.

Expression of adenovirus mRNA from early genes requires the E1A gene product (reviewed in Kovesdi et al., 1986a). In addition, transcription of all the early mRNAs on the viral chromosome is regulated in cis by the E1A transcriptional enhancer (Hearing and Thomas, 1986). It therefore seems likely that the viral early genes form a gene battery, which is coordinately regulated by the E1A product, and the E1A gene's enhancer element.

In trans, the E1A gene product enhances levels of other early mRNAs by enhancing the levels of a cellular factor which binds to adenovirus DNA (Kovesdi et al., 1986a,b). To date, the cellular factor has been shown to bind 5' to early gene E2, and to regulate its transcript levels (*ibid*). The E2 5' regulatory sequences bound by the cellular factor have been localized to the region from -74 to -33.

The DNA sequence of the region surrounding this binding site is depicted below.

```

                -60                -40                -40
      GACGTAATTTT  CGCGC TTAAA ITGAGAAAGG  CGCGCAAA CTAATC
      _____
                -20                +1
      CTTAAGAGTCAGCGCGCAGTATTTGCTGAA
  
```

(from Kovcsdi *et al.*, 1986).

The numbering is relative to the mRNA 5' end (underlined sequences have homology with the E1B gene). Essentially, the sequence is a dyad (arrows), capable of forming a stem loop, as was the case for the yeast histone gene CCR sequence. Contained within the sequence is a purine-rich (12/17 bp) region bounded by CGCG repeats. Deletions initiating upstream and extending into this regulatory region so as to remove one of the two repeats drastically reduces both E2 mRNA and binding of the E1A-regulated DNA binding factor.

Although DNA binding studies similar to those reported for the E2 gene have not been performed with the periodically expressed E1B gene, DNA sequence analysis shows some striking similarities within their regulatory regions. The E1B regulatory region shown below contains sequences that clearly resemble those found within the binding sites found upstream of E2. Both genes have 5' regions containing repeated copies of an element having the consensus CGCGTTAAA. Hence, this element may be responsible for the E1A-dependent regulation of these two "early" virus genes.

Depicted below is the end of the E1A gene and the start of the E1B gene (from the Ad5 sequence reviewed in Tooze, 1980).

E1A 3' end -50

TAATAAA GGGTGAGATAA TGTTT AACTTGCATG GCGTGTTAAA TGGGG

-30

CGGG GCITAAA GGG TATATAAT GC GCC GTGGGCT AATCTTGCTT

+1

ACATCTGACCTC ATG

mRNA start E1B start

The sequences underlined are homologous to the E2 promoter region (sequenced by Gingeras *et al.*, 1982) which binds the E1A gene product.

The adenovirus E1A and E1B RNAs are separated by about 80 bp. The E1A AATAAA consensus transcription "termination" sequence (reviewed in Nevins, 1983) precedes the 3' E1A RNA terminus, and the TATA box for E1B lies within 40 bp of this sequence, followed within 25 bp by the E1B 5' RNA end at +1, and finally the E1B translational start codon.

Lastly, the CCGG motifs are repeated several times in the 5' region of both E1B and E2 (Murthy *et al.*, 1985; Kovesdi *et al.*, 1986). This redundancy may explain the inability of linker scanning mutations to detect a specific regulatory element 5' of E2 (Murthy *et al.*, 1985).

In contrast to E1B, the HSP70 gene contains little homology to the E2 gene within its 5' region (Hunt and Morimoto, 1985). The lack of sequence homology probably reflects the fact that the timing of HSP70 expression is very different from that of E1B and E2 (Kao *et al.*, 1985). For example, E1A, E2 and HSP70 RNA levels all fluctuate periodically. However, the periodic fluctuation of HSP70 RNA appears to be regulated mainly at the

level of transcription, while E1A and E1B RNAs are also regulated at the level of RNA stability.

In summary, the levels of RNA from several mammalian genes appear to be cell cycle regulated. The fluctuations observed can result from transcriptional, and/or post-transcriptional regulation. The timing of the periodic expression of the adenovirus E1B gene's mRNA is associated with the presence of a particular sequence of DNA present in its 5' flanking region, and a similar sequence is present within the upstream region of the E2 gene. The latter may be regulated in a similar fashion to E1B, since both genes require the E1A gene product for their expression. The sequence 5' of the E2 gene binds a cellular product which is elevated in the presence of the viral E1A gene product. Since the latter is the transforming factor for adenovirus oncogenesis (reviewed by Tooze, 1981), the E1A product must be capable of stimulating the transformed cells to cycle, and so will either directly or indirectly cause cellular CCR genes to be expressed.

A.3. The START Concept.

Nasmyth (1985b) assumed that the cell-cycle START (cdc28-4) - dependent regulation of the yeast HO gene was periodic, as did Hereford et al for the cdc28-4 - dependent regulation of the yeast IRT genes. In fact, of the regulatory elements tested, only the histone CCR element was shown to cause fluctuations in RNA levels beyond the first generation after arrested cells had been stimulated to proliferate. It was shown that the histone "16mer" and HO CACGAAAA were capable of transiently enhancing expression of these genes in cells stimulated to proliferate from an arrested state. Yet, the lack of second-generation evidence demonstrating periodic expression leaves open the possibility that the latter two elements do not regulate periodic fluctuations in mRNA levels, but only "proliferation" dependent changes which occur when cells escape from an arrested state.

This ambiguity also appears in many studies of mammalian genes associated with the cell cycle. These studies depicted mRNAs which were elevated shortly after cells are stimulated to proliferate in culture (reviewed by Marcus, 1985; Denhardt, 1986). Again, most of these studies did not investigate subsequent cell cycles to determine whether the gene's expression was truly periodic.

It is important to attempt to resolve this difficulty, because many genes in addition to CCR genes may be activated when cells are released from an arrested state.

Part of the problem is technical, since synchronous cell populations released from naturally arrested states are more easily studied than single cells or cells fractionated by cell-cycle stage. Kao et al (1986) may have uncoupled cell cycle regulation from proliferation-dependent regulation by synchronizing cells at the G1/S boundary (a common

technique for mammalian cell-cycle studies), at which time cells are already committed to the cell cycle. Such a "system" may therefore be better suited to studies of periodic expression. However, there are two problems associated with this method. One, the buildup of mRNA or gene product may be an artifact, caused by the cell cycle block, and not by periodic expression. Two, genes which are expressed after START (R) but before S (like TMP1, CDC8, CDC9, H2A and H2B, and H0, are already expressed by this time.

Another aspect of the problem is semantic. For this reason, I will here relate the concept of START, a term developed in yeast, to the mammalian "restriction point", as these terms apply to cell cycle regulated genes.

A.3.1. START in yeast versus "R" in animal cells.

START will be defined as a point or stage in the yeast cell cycle shortly before S phase the passing of which commits a cell to complete one division cycle. A START-dependent gene is one whose expression, requires the completion of START. This definition contrasts with that of a CCR gene, since periodic fluctuations in mRNA or gene product are inevitably affected by each completion of START. By this definition, all CCR genes are START-dependent, but START-dependent genes need not be CCR genes.

One advantage of this definition of START is that it corresponds well to the definition applied to the mammalian restriction point, R (reviewed by Denhardt, 1986). That is, cells which have progressed past R are committed to another cell cycle. However, the definition of R in mammalian cells goes further: the cell will complete the cell cycle even if protein synthesis is blocked (ibid), unlike yeast (eg. Hartwell, 1974; G. Johnston, pers. comm.).

Another advantage of the definition is that the events critical to START need not be confined to the G1 interval. START itself can occur before mitosis in yeast cells recovering from cell cycle constraints such as growth in hydroxyurea, which inhibits ribonucleotide reductase, or in cdc8 cells at a semi-restrictive temperature (Singer and Johnston, 1983). This implies that, for cells in balanced growth, the amount of time a cell spends in G1 before passing START depends on whether the cell has accumulated the wherewithal to do so in the previous cell cycle (including components required for DNA synthesis).

Third, this definition of START can be reconciled with the data cited above, as follows.

A.3.2. CDC28, α -factor and START.

Hartwell (1974) defined START as that stage in G1 at which cells were arrested by α -factor or by the cdc28 mutation. This stage was considered to be "late" in G1, but as pointed out above, this definition would depend on the length of G1.

A cell in rapid growth would have to be arrested by α -factor immediately following mitosis, since G1 is short. This predicts that α -factor acts during the cell cycle prior to arrest. A cell with a long G1 could grow before reaching the block. For example, daughter cells, which following cytokinesis are smaller than mother cells normally have a longer G1 due to a minimum size requirement needed for passing START. Daughter cells achieve "readiness" equal to mother cells if held in α -factor at START (proposed by Singer and Johnston, 1983). This apparently results because cells continue to grow in the presence of α -factor, which allows daughter cells to attain the minimum size requirement.

In contrast to α -factor-induced first-cycle arrest, cdc28-4 mutants may undergo several cell cycles prior to arresting at START at the restrictive temperature (reviewed by Camonix, 1986). How can this observation be reconciled with the mutant's inability to escape from G_0 arrest (Nasmyth, 1983, 1985a,b) in terms of ability to pass START?

A.3.3. Yeast "START" mutants versus START.

Many mutations besides the cdc28-4 mutation cause cells to arrest at START. However, there is no indication that these genes are directly involved in the passing of START (ie. initiating a DNA synthetic cycle). This is probably true of most or all START mutants (defined as mutants which arrest at START). Instead, these mutations may all be in genes essential to the cell, and affect the cell with different severities depending on the "leakiness" of the mutation.

START mutants have been divided into Class I and Class II (Reed *et al.*, 1985; Camonix *et al.*, 1986), based on the phenotypes of conditionally lethal mutants arrested under restrictive conditions. Class I mutations (eg. of CDC28 encoding a kinase, CDC36, CDC37 and CDC39) cause a G_1 arrest which resembles the arrest induced by the mating pheromone α -factor (Reed, 1980). Class I mutants can still increase in cell volume and mate under restrictive conditions, and synthesis of many mRNAs and proteins occurs in the arrested cells.

Class II START mutations (eg. of CDC19 encoding pyruvate kinase, CDC25 encoding a regulator of adenylate cyclase, CDC33, or CDC35 encoding adenylate cyclase) cause arrest in a G_1 stage which resembles G_0 . Class II mutations do not synthesize RNA or protein, and neither mate nor grow under restrictive conditions.

Note that both classes of START mutation arrest cells in the unbudded

state, but Class II mutations appear to be more severe. Although the nature of the Class II "G₀"-like phenotype is undefined, it is characteristic of an undernourished state of the cell. In contrast, the unbudded, but active, state of α -factor- or cdc28-4-arrested cells more clearly defines a state of readiness-to-divide on the part of the cell. This view is supported by the phenotypes of yeast prt1 mutants (Hartwell and McLaughlin, 1969). These mutants are temperature-sensitive-lethal, and were originally characterized as defective in the initiation of protein synthesis. At 36°C, mutant cells arrest immediately without regard to cell cycle stage (G. Johnston, pers. comm.). At 34°C the cells arrest in G₁ displaying a Class II START mutant phenotype. At 32°C the mutants resemble Class I START mutations.

The prt1 mutation therefore suggests the probable true nature of many START mutants, as "leaky" mutants in essential genes which are able to complete a cell cycle by virtue of their leakiness. Upon completion of mitosis, the more stringent Class II mutants seem to be more severely nutritionally depleted and enter G₀. The more "leaky" Class II mutants would be able to reach, but not pass, START.

Note that if most START mutations are in essential genes, their expression early in the cell cycle would be expected, and should not implicate them in cell cycle regulation. In contrast, a class of animal genes, oncogenes (Denhardt et al., 1986) may constitute bona fide START genes. Many viral oncogenes (v-onc) confer on oncogenically transformed cells the inability to arrest properly at "R" (ibid). The mRNAs from these genes' cellular equivalents appear in the first G₁ in cultured cells stimulated to proliferate (ibid, Figure 1.1), and so could be associated with a START-like signal. Furthermore, expression of these genes in the G₁ of proliferation-stimulated cells appears to be ordered,

which suggests that events occurring early in G1 may be required for subsequent G1 events, such as the passing of "R".

A.3.4. START-dependent genes versus CCR genes.

It should be recalled that CCR genes are defined as genes whose expression fluctuates periodically during the cell division cycle. Implied in this definition is that the cells are in balanced growth, and not initiating or terminating proliferation.

It is possible that START-regulated genes are expressed constitutively in cells which are dividing rapidly, but are repressed when cell division is inhibited. Such repression may lead to a compensatory overexpression of the START-regulated gene when proliferation is resumed. This means that START-dependent gene expression, elevated by START-dependent synchronizing techniques such as nutritional or α -factor blocks, may peak during the first cell cycle following arrest, even though the gene is not periodically expressed. A possible example of a gene which is START regulated, but not periodically expressed at the mRNA level is the yeast DCDI gene (McIntosh and Haynes, 1986).

A.4. Cell cycle regulation by CCR genes: the "unstable protein model".

The length of the cell cycle is normally determined by the length of the G1 stage in mammalian cells and in budding yeast, whereas S phase, G2 and mitosis intervals are normally of fixed duration (Mitchison, 1971). What controls the length of G1? Furthermore, why should increasing the length of the cell cycle change the length of G1 prior to START, without altering the length of other cell cycle stages (reviewed by Carter *et al.*, 1983)? The most attractive hypothesis presented to date proposes that levels of

a labile protein must reach a critical threshold level before commitment to cell division (Pringle and Hartwell, 1981; Coppock and Pardee, 1985; early references were reviewed in Mitchison, 1971).

According to this model, a labile protein called the "trigger protein" is responsible for completing the START event (reviewed by Albert et al., 1983). Cells cannot complete START unless this labile protein reaches a threshold concentration. Because of its instability, the hypothetical protein would only accumulate in sufficient quantities to stimulate a cell division cycle if it was synthesized relatively rapidly. As soon as its synthesis is curtailed (at some point between START and the following mitosis), levels of the protein are then proposed to decrease at a constant rate as the cell cycle progresses towards the following START. When the cell proceeds through the cell cycle to the next G1 the labile protein is again synthesized.

The rate of synthesis of the labile protein is further proposed to vary directly with the overall metabolic rate of the cell. Therefore, the time required for the labile protein to accumulate to the threshold concentration required to complete START (ie. the length of G1 prior to the completion of START) will vary depending on its rate of synthesis.

Could the aforementioned cell cycle regulated enzymes involved in DNA synthesis constitute "trigger proteins"? Several lines of evidence suggest that this may be the case. First, like the hypothetical "trigger protein", several genes whose products are required for DNA precursor synthesis or DNA synthesis itself are cell cycle regulated, and their cell cycle dependent expression occurs during G1 at or very close to START (section A.2.3.). Second, levels of the trigger protein should be present at a concentration near threshold levels in log phase cells. At least one of the CCR gene products, TS, has been shown to be present at intracellular

levels, near those, which would make dTMP ~~rate~~ limiting for DNA synthesis (Bisson and Thorner, 1981). Third, the trigger protein should be unstable. Interestingly, the yeast TS and DNA ligase CCR gene products have both been shown to be far more labile than bulk cellular protein (Greenwood et al., 1986; and White et al., 1986; respectively). Fourth, a "pre-replication complex" of several replicative enzymes was observed associated with yeast 2 μ -circle DNA by Jaswinski and Edelman (1984). This observation suggests that the assembly of the multi-enzyme complex precedes S phase and so may be required for its onset. Several genes whose products are found in replication complexes (Reddy, 1977) have been shown to be CCR genes in yeast, based on the periodic fluctuations of their transcripts (including TS, DNA ligase and dTMP kinase, section A.2.3.; and DNA polymerase, J. Campbell, pers. comm.).

Therefore the rate of accumulation of gene products required for the formation of a pre-replication complex could determine the length of time spent in G1 before completion of START. This in turn implicates the cell cycle dependent regulation of expression of CCR genes like TMP1 in initiating a cell division cycle, and in determining the rate of cell division.

A.5. Introduction to the yeast TMP1 gene.

Interestingly, thymidylate synthase (TS), an enzyme associated with DNA synthesis and with (pre-) replication complexes (Reddy, 1977) shows periodic instability in yeast (Storms et al., 1984). Greenwood et al. (1986) proposed that levels of TS also fluctuated in direct proportion to the rate of cell division, and there is every reason to assume that RNA levels may be similarly regulated (Calmels, in preparation). In addition, TS mRNA also fluctuates periodically during the cell cycle (Storms et al.

1984). The dependence upon START for the elevation of both TS and its mRNA led us to conclude that IMP1, the gene encoding TS, was a START-dependent CCR gene.

TS catalyzes the de novo synthesis of deoxythymidylate (dTMP) by replacing the hydrogen at ring position 5 of the precursor deoxyuridylate (dUMP) with a methyl group from N⁵,N¹⁰-methylenetetrahydrofolate (early references were reviewed by Blakely (1969).

The TS-encoding IMP1 gene has been well characterized genetically prior to this study (Brendel and Langjahr, 1974; Little and Haynes, 1979; Kunz, 1982). The reaction catalysed by TS is a target for cancer chemotherapy (Damenberg, 1977). Imbalances in the dTMP pools have been shown to affect cell viability, mutation rates and recombination rates (reviewed by Barclay *et al.*, 1982; Haynes and Kunz, 1986; and by Taylor, 1986). The TS gene product has been characterized by Bisson and Thorner (1981). The gene was also of interest to investigators of eucaryotic DNA synthesis, who created dTMP auxotrophs for the purposes of DNA labelling experiments in yeast (Brendel and Fath, 1974; Little and Haynes, 1979). This first required the isolation of tup mutants which were permeable to dTMP supplementation (Brendel and Haynes, 1972; Little and Haynes, 1979). One TS mutant is the tmp1-6 allele of IMP1. Strains harbouring this mutation are auxotrophic for thymidylate. In a tup background, yeast containing this mutation can grow in complete or minimal medium supplemented with 100 ug/ml of dTMP.

The tmp1-6 and cdc21-1 (at 37°C) alleles cause arrest of the DNA replication apparatus during S phase, which leads to the arrest of the parallel budding cycle just prior to cytokinesis. The "dumbbell" morphology displayed is characteristic of mutants which are defective in DNA synthesis (Game and Johnson, 1978), including the aforementioned cdc8

Figure 1.3. Sequence of the yeast TMPI gene (the non-coding DNA strand, adapted from Taylor, 1986, starting at the HindIII restriction site at -377).

Bases are numbered on the left of the Figure from the 5' HindIII site, and, on the right of the Figure, relative to the first of two putative translational start sites. The single letter code for the putative polypeptide product is given below the DNA sequence.

Restriction sites relevant to this thesis are shown above the DNA sequence. The large repeat (ibid) 5' to the coding region is overlined. This repeat has homology with other COCO repeats (see text). Other putative regulatory sequences (underlined) are, TATA and AATAAA elements correlated in other studies with mRNA 5' ends and 3' ends, respectively, and a cryptic intron consensus (ibid) located at +99 (GGTACGT), +163 (TACTAAC) and +189 (TAG).

1 HindIII
 AAGCTTCTTTCCCTCTCGTCTGTAAAGGACGGGATTGAGATTCAATTAAAAATTAGTTTTATTG6AAAA -305
 73 PvuII
 GGCCAATAAAATATGCAAAGGTCCCGTCATTATACTGCAGCTACCTCAAAGAGATTTAGCTTTGTGTCTCA -233
 145
 ACGTAGGACCGCATTGAACAGGTTGGAAGAGGTTGCTTTTTTGACCTTCTTCTCAAACCTAATATTTGGT -160
 218 MluI MluI
 GACCGTTAAATAGAAAAAATGAAAAAGACCTTAATTGACCGCTTCTGAAATATTTACTATAACATAGC -89
 289
 TGTAAATTGATTTTCTAAACTTGTAAAGTACTGTGTATATCAAACAATAAAGTATCAAGGAGAGAGCTTCA -18
 360 +1 SmaI
 TAACAGAACGGTACAGG ATG ACT ATG GAC GGA AAA AAC AAA GAA GAA GAG CAA TAT CTT GAT +45
 M T M D G K N K E E E Q Y L D
 422
 CTA TGC AAA AGA ATC ATT GAT GAA GGT GAA TTT AGG CCA GAT AGA ACA GGC ACT GGT ACG +104
 L C K R I I D E G E F R P D R T G T G T
 482 PvuII
 TTG AGT CTT TTT TGC ACC ACC CAG CTG CGT TTT AGT TTA CGT GAT GAT ACC TTC CCA CTA +164
 L S L F C T T O L R F S L R D D T F P L
 542
 CTA ACG ACC AAG AAG GTT TTT ACT AGA GGT ATC ATA CTT GAA CTA TTG TTG TTT TTG GCC +224
 L T T K K V F T R G I I L E L L L F L A
 602
 GGT GAT ACA GAC GCT AAC CTA TTG TCT GAG CAA GGT GTT AAG ATT TGG GAC GGT AAT GGA +284
 G D T D A N L L S E Q G V K I W D G N G
 662 BglII
 TCT CGT GAA TAT TTA GAT AAG ATG GGG TTC AAA GAT AGG AAA GTA GGA GAT CTG GGG CCC +344
 S R E Y L D K M G F K D R S V G D L G R
 722
 GTT TAC GGA TTT CAA TGG AGG CAT TTT GGT GCT AAA TAC AAG ACG TGC GAT GAC GAC TAT +404
 V Y G F Q W R H F G A K Y K T C D D D Y
 782
 ACT GGA CAA GGT ATT GAT CAA TTG AAA CAG GTT ATA CAT AAA CTA AAG ACA AAT CCC TAC +464
 T G Q G I D Q L K Q V I H K L K T N P Y
 842
 GAT AGA AGA ATC ATT ATG AGT GCC TGG AAC CCA GCT GAT TTT GAC AAA ATG GCT TTG CCG +524
 D R R I I M S A W N P A D F D K M A L P
 902 BamHI
 CCA TGC CAT ATT TTT TCA CAG TTC TAT GTC AGT TTC CCC AAA GAA GGA GAA GGA TCC GGT +584
 P C H I F S Q F Y V S F P K E G E G S G
 962
 AAG CCC CGC CTT TCA TGC TTA TTG TAC CAA CGT TCT TGT GAT ATG GGG TTG GGA GTA CCA +644
 K P R L S C L L Y Q R S C D M G L G V P
 1022 SmaI
 TTT AAC ATT GCA TCA TAT GCT TTG TTG ACT AGA ATG ATT GCT AAA GTT GTC GAC ATG GAG +704
 F N I A S Y A L L T R M I A K V V A M E
 1082
 CCA GGT GAG TTC ATT CAT ACT TTG GGG GAT GCA CAT GTT TAT AAA GAC CAC ATT GAT GCC +764
 P G E F I H T L G D A H V Y K D H I D A
 1142
 TTG AAA GAA CAA ATC ACC AGA AAT CCA AGA CCA TTC CCG AAA CTA AAG ATT AAA AGG GAC +824
 L K E Q I T R N P R P F P K L K I K R D
 1202
 GTT AAA GAC ATT GAT GAT TTC AAG TTG ACA GAC TTT GAA ATT GAA GAT TAT AAT CCT CAT +884
 V K D I D D F K L T D F E I E D Y N P H
 1262 EcoRI
 CCA AGA ATT CAA ATG AAA ATG AGT GTA TAA TTGACAGCACGAGGAGAAACAAATCATGTACTT +948
 P R I Q M K M S V ***
 1326 ACGTATATACACACAAAAATCGAATGTATATAATACAATGGTTTATTATGCAAAAAATATAGAA +1010
 1388 ATTATTAAGACACAGGCTAAAAAGACTCATATATCATTTTTTGGTGCATATGTTTTTGGTTG +1074
 1452 GATGTTTCACTGCCCTTACCATTTCCAAATAAATCAAATACTGCTAAATCATCTTCATTTAAGT +1130

and cdc9, IMP1 is not an "essential" gene (one whose gene product must be physically present, even if mutated), since deletions of the gene do not produce inviable progeny, merely auxotrophs for thymidylate (G. Taylor, 1986).

Originally, our laboratory's interest was in the coordinate transcriptional and post-translational regulation of IMP1 as it pertained to nucleotide metabolism. TS was shown to accumulate periodically near the beginning of S phase (Storms *et al.*, 1984). This periodic accumulation was determined, in part, at the level of the transcript. Of other genes involved in pyrimidine metabolism only the dTMP kinase gene CDC8 introduced above has since been shown to be periodically expressed at the RNA level. Three other genes in pyrimidine DNA precursor metabolism, DCD1, DUT1 and DFR1 (encoding dCMP deaminase, pyrophosphatase and dihydrofolate reductase, respectively) encode RNAs which are not periodically expressed (McIntosh *et al.*, 1986), although DCD1 expression may be START-dependent.

The IMP1 gene, including more than 400 bp of 5' information had earlier been cloned on a yeast plasmid, pTL1 (Taylor *et al.*, 1982), and the cloned gene complemented the tmp1-6 and cdc21 auxotrophies. This result indicated that the cloned gene, contained on a HindIII restriction fragment (ibid) was the correct gene, and contained sufficient regulatory controls to function in yeast. The gene was subsequently sequenced (Figure 1.3), and S1 mapping suggested that the 5' IMP1 mRNA end mapped roughly 60 bp 5' to the initiation codon (Taylor *et al.*, 1986). The predicted structural (coding) portion of IMP1 extends 906 bp from the ATG (+1), and the first 14 codons are in agreement with the first 14 amino acids of the yeast TS protein, as sequenced by Bisson and Thorner (1981).

The gene contains several other putative regulatory sequences common to other eucaryotic and yeast genes. These include "TATA" sequences at -280, -105 and -59, and at positions 946, 968, 998 and 1035, just downstream of the coding region. The structural portion of the gene contains a consensus glycosylation site (Asn-Gly-Ser at position +273) and a cryptic intron (G⁺GTACGT at position +93; TACTAAC at +157; TAG at +182). This intron is not excised from the bulk of IMP1 RNA, based on S1 protection studies (Taylor et al, 1986). Consensus mRNA "stop" signals (AATAAA) are found at +1093 3' to the coding region, as well as at -38 and at -140 and -300 5' to the coding region. The 5' locations of these putative transcription termination signals reinforces the emerging implication of transcriptional stop sequences in 5' transcript regulation (Yarger et al, 1986; see above).

A.6. Studying TS regulation using lacZ gene fusions. Having ascertained that IMP1 was cell cycle regulated, we set out to study how the expression of the gene, its transcript and its product were modulated. To do this, we decided to fuse the IMP1 5' information to the structural portion of the E. coli lacZ gene. The β -galactosidase gene product of lacZ is not required for growth and is readily detectable. The resulting gene fusion could then be introduced into yeast on a multicopy plasmid so as not to perturb the chromosomal IMP1 gene. Once the fusion gene was shown to be capable of expressing β gal activity in yeast the system could then be used to study IMP1 expression.

"Translational" gene fusions of the type used here generally contain upstream regulatory sequences, together with a small part of the structural gene, fused in-frame to the coding region of the lacZ gene. Such gene fusions direct the synthesis of active β -galactosidase in

yeast. Furthermore, the presence of one to several hundred amino-terminal peptides (from the upstream gene) fused to the *Bgalactosidase* peptide (Kania and Muller Hill, 1977; see reviews by Rose and Botstein, 1983; Guarente, 1983 and Casadaban *et al.*, 1983) does not affect the encoded *Bgal* activity.

Such "Yeast promoter- 'lacZ'" translational gene fusions have been used to map regulatory information 5' to a number of yeast genes, in addition to TRT and HO as described above. Upstream regulatory sequences from CYC1 (Guarente and Ptashne, 1980; Guarente, 1983; Guarente *et al.*, 1983), GAL1 - GAL10 (Guarente *et al.*, 1982; West *et al.*, 1984), GAL7 (Yarger *et al.*, 1986), LEU2 (Martinez-Arias and Casadaban, 1983), HIS4 (Silverman *et al.*, 1982) and GCN4 (Hinnenbusch, 1986), to name a few, have all been studied using lacZ fusions.

Although the studies cited above generally sought to explain the regulation of the fusion gene at the level of transcription, the technique can also be used to detect post-transcriptional regulation. For example, the same Hinnenbusch (1986) study cited above used GCN4'-'lacZ' fusions to show that large amounts of product could be expressed even when transcript levels were low, and vice versa. As a result, this author was one of the first to confirm the aforementioned possibility that gene expression could be post-transcriptionally modulated at the level of translation.

As a result of the ability to detect transcriptional, translational and post-translational regulation, lacZ fusions provided a powerful means of determining the level(s) at which IMP1 expression was periodically modulated. Following the detection of periodic alterations in fusion-encoded *Bgalactosidase* ("Bgal") activity, RNA from the fusion could be further tested to distinguish the level of control; for periodic

transcription via incorporation studies, for periodic fluctuations in RNA levels via Northern analysis, for periodic instability of fusion RNA by comparing the first two parameters, and for transcript 5' ends, multiple transcripts and RNA processing via primer extension and S1 mapping.

Post-translational control of TS has already been studied via these IMP1'-lacZ translational fusions. By using larger or smaller segments of the IMP1 coding region fused to lacZ, different-sized fusion proteins were created. Greenwood (1986) correlated the stabilities observed for particular thymidylate synthase'- β -galactosidase fusions with TS instability under conditions which curtail metabolic rates in yeast, and proposed that both TS and the fusion-gene product were unstable due to a 50 a.a. sequence in TS. Poon (in preparation) showed that the unstable thymidylate synthase'- β -galactosidase fusion gene products (and, by inference, TS) were non-randomly distributed in the cell.

This study is divided into four sections. The first briefly describes the cell cycle dependent regulation of IMP1, and demonstrates that IMP1 is both a OCR and START-regulated gene. The second describes the construction of a series of plasmids carrying 5' IMP1 regulatory sequences fused to lacZ to produce IMP1'-lacZ fusion genes. The third characterizes the expression of these fusion genes in yeast. A final section briefly compares RNA transcripts expressed from IMP1'-lacZ fusions with their β -galactosidase activities. The study tests the following hypotheses: that the periodic expression of IMP1 is controlled by its 5' region; that the aforementioned region associated with OCR genes is in fact a OCR element; that the regulation of the IMP1'-lacZ fusion derivative is START-dependent; and finally that the regulation of fusion transcripts is identical to that of IMP1 mRNA.

B. MATERIALS and METHODS.

B.1. Strains and plasmids. The strains and plasmids used in this study are given in Tables 1 and 2, respectively. Most experiments in yeast were performed with yeast strain AH22, while plasmid manipulations used *E. coli* strain JF1754 as a host. When referring in the text to strains which contain plasmids, the plasmid name precedes the strain name. For example pRS269-AH22 designates yeast strain AH22 transformed with plasmid pRS269. Nomenclature for the IMP1'-lacZ fusion genes follows that of Guarente and Ptashne (1981). Here, "IMP1'" indicates that upstream sequences of this gene are present. "-lacZ" indicates that all natural 5' regulatory elements have been deleted, such that only sequences which encode the structural β -galactosidase (" β gal"), and sequences 3' to the gene, are present.

B.2. Media.

B.2.1. Yeast Media. Complete medium for the growth of yeast was YEP, consisting of 2% Bacto-peptone, 1% yeast extract in water (all W/V), or YEPD with dextrose included 2%. For minimal medium, 0.67% YNB (Difco yeast nitrogen base) replaced the peptone and yeast extract, and amino acid requirements were added to 50 μ g/ml.

For asynchronous growth studies and for studies of α -factor mediated arrest in culture, the minimal medium "2 x YNBH, 1x D" (1.34% YNB, 100 μ g/ml histidine, 2% glucose) was used except where noted. This medium is referred to as selective medium in the text, since, without leucine supplementation, cells were required to maintain the replicating plasmids studied here, which contained the yeast LEU2 gene except where noted. When required, this medium was also supplemented with 100 μ g/ml

Table 1.A. *E. coli* Strains

<u>E.coli</u> host Name	<u>Derivative</u> lab number	<u>Plasmid</u> Borne	<u>Genotype</u>	<u>Source</u>
JF1754	RS3	none	<u>hsdr₊ lacZ⁻ gal⁻ metB⁻ leuB⁻ hisB436</u>	J. Friesen
	RS58	pRS58	as above except <u>LEU⁺ amp^r</u>	R. Storms
	RS93	pYF91	as above except <u>LEU⁺ amp^r</u>	R. Storms
	RS222	pTL1	as above except <u>LEU⁺ amp^r</u>	R. Storms
	RS231	pRS231	as above except <u>amp^r</u>	R. Storms
	RS258	pYT760-RYP3	as above except <u>LEU⁺ amp^r</u>	D. Thomas
	RS264	pRS264	as above except <u>LEU⁺ amp^r</u>	This study
	RS269	pRS269	as above except <u>LEU⁺ amp^r</u>	This study
	RS306	pRS306	as above except <u>LEU⁺ amp^r</u>	L. Lee
	RS479	pRS479	as above except <u>amp^r</u>	P. Poon
	RS486	pMC931	as above except <u>amp^r</u>	M. Casadaban
	RS535	pRS535	as above except <u>LEU⁺ amp^r</u>	This study
	RS537	pRS537	as above except <u>LEU⁺ amp^r</u>	This study
	RS593	pRS593	as above except <u>LEU⁺ amp^r</u>	E. Calmels
	RS665	pRS665	as above except <u>amp^r</u>	This study
	RS669	pRS669	as above except <u>LEU⁺ amp^r</u>	This study
	RS676	pRS676	as above except <u>LEU⁺ amp^r</u>	This study
	RS725	pTL30	as above except <u>LEU⁺ amp^r</u>	G. Taylor
	RS726	pTL31	as above except <u>LEU⁺ amp^r</u>	G. Taylor

(continued)

Table 1.A. *E. coli* strains (continued)

<u>E. coli</u> host Name	<u>Derivative</u> lab number	<u>Plasmid</u> Borne	<u>Genotype</u>	<u>Source</u>
JF1754	RS727	pTL32	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	G. Taylor
	RS728	pTL33	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	G. Taylor
	RS729	pTL35	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	G. Taylor
	RS730	pTL34	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	G. Taylor
	RS731	pRS479-10	as above except <u>amp</u> ^r	This study
	RS740	pRS593-1	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	This study
	RS741	pRS535-1A	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	This study
	RS742	pRS535-7A	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	This study
	RS743	pRS535-10A	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	This study
	RS744	pRS535-10C	as above except <u>LEU</u> ⁺ <u>amp</u> ^r	This study
LG90	RS769	pLG669-Z	Δ lacpro	L. Guarente

Table 1b. Yeast Strains

Host Name	Derivative lab number	Introduced plasmid	Genotype	Source
LL20	RS7	none	<u>MATα, leu2-3,2-112, his3-11,3-15</u>	G. Fink
S288C	RS29	none	<u>MATα, SUC2, mel, mel, gal2, CUP1</u>	Stock center
AH22	RS224	none	<u>MATα, leu2-3,2-112, his4-514</u>	G. Fink
	RS290	pRS58	same as AH22, except <u>LEU⁺</u>	This study
	RS294	pYT760-RYP3	same as AH22, except <u>LEU⁺</u>	This study
	RS296	pRS264	same as AH22, except <u>LEU⁺</u>	This study
	RS458	pRS306	same as AH22, except <u>LEU⁺</u>	L. Lee
	RS574	pRS269	same as AH22, except <u>LEU⁺</u>	This study
	RS575	pRS535	same as AH22, except <u>LEU⁺</u>	This study
	RS577	pRS537	same as AH22, except <u>LEU⁺</u>	This study
	RS898	pRS669	same as AH22, except <u>LEU⁺</u>	This study
	RS900	pTL30	same as AH22, except <u>LEU⁺</u>	This study
	RS902	pTL31	same as AH22, except <u>LEU⁺</u>	This study
	RS903	pTL32	same as AH22, except <u>LEU⁺</u>	This study
	RS904	pTL33	same as AH22, except <u>LEU⁺</u>	This study
	RS906	pTL34	same as AH22, except <u>LEU⁺</u>	This study
	RS908	pTL35	same as AH22, except <u>LEU⁺</u>	This study
	RS910	pRS535-1A	same as AH22, except <u>LEU⁺</u>	This study
	RS912	pRS535-10C	same as AH22, except <u>LEU⁺</u>	This study
	RS914	pRS535-10A	same as AH22, except <u>LEU⁺</u>	This study
	RS915	pRS535-7A	same as AH22, except <u>LEU⁺</u>	This study
	RS917	pRS676	same as AH22, except <u>LEU⁺</u>	This study
AH22	RS934	pRS665	same as AH22, except <u>LEU⁺</u>	This study
X2180-1A	RS252	none	<u>MATα</u>	Stock center
DBY747	RS599	none	<u>MATα, leu2-3,2-112, his3Δ1, ura3-52, trp1-289</u>	Stock center

Table 2. Plasmids

Plasmid Name	Pertinent features	Source
PMC931	pACYC177 ori _{amp^r} ; Km ^r ; E. coli lacZ _{YA} (9.9 Kb) ^a	Casadevall et al (1983) <i>ibid.</i>
PMC1871	pBR322 ori _{tet^r} ; lacZ (7.8 Kb)	
PFY91	pBR322 ori _{amp^r} ; yeast 2 μ ^b and LEU2 (13.4 Kb)	Storms et al (1979) R. Storms
PRS58	pBR322 ori _{amp^r} ; lacZ _{YA} ; yeast 2 μ and LEU2 (20.3 Kb)	
PL1	pBR322 ori _{amp^r} ; yeast 2 μ , LEU2 and IMP1 (23.4 Kb)	Taylor et al (1982) R. Storms
PRS231	pBR322 ori _{amp^r} ; IMP1'-lacZ _{YA} and 2 μ (15.7 Kb)	See text
PRS264 ^c	pBR322 ori _{amp^r} ; IMP1'-lacZ _{YA} ; 2 μ and LEU2 (17.9 Kb)	D. Thomas
PY1760-RYP3	pBR322 ori _{amp^r} ; RYP3'-lacZ _{YA} ; 2 μ and LEU2 (17.2 Kb)	Gargente and Plishne (1981) P. Poon
PL0669-Z	pBR322 ori _{amp^r} ; CYC1'-lacZ; 2 μ and URA3 (11.8 Kb)	E. Calmels
PRS479	pBR322 ori _{amp^r} ; yeast URA3 and IMP1 (9.7 Kb)	
PRS593	pBR322 ori _{amp^r} ; yeast LEU2 and IMP1 (9 Kb)	
YIP5	pBR322 ori _{amp^r} ; yeast URA3 (5.6 Kb)	Struhl et al (1979)

^a The E. coli nomenclature for protein fusions is used in this study.

^b "2 μ " denotes the 3.9 Kb fragment of the yeast 2- μ m circle.

^c Plasmid PRS264 is identical to PRS269 and to PRS535 except for the IMP1' sequence differences shown in Figure 3B.

leucine. Solid media (plates) contained 2% agar. It should be noted that the glucose concentration was different from the 4% used by Greenwood (M.Sc. thesis, 1986); differences in growth rates and β -galactosidase enzyme activities due to the glucose concentrations are given in Figures 17A and B.

For synchrony experiments (see below) the 2 x YNBH, 1 x D medium also contained 10% YEP medium (V/V), with glucose added to 2%. This "0.1% YNB" (Hereford and Osley, 1981) medium was generally prepared and prewarmed to 30°C just prior to use. This medium is referred to as synchrony medium in the text, and is non-selective for the plasmids studied here. For this reason, caution should be exercised in comparing absolute β gal. activity levels from different plasmids between synchrony experiments (refer to Tables 2 or 4 for values obtained with the plasmids under selection).

B.2.2. *E. coli* Media. "YT" was the complete medium for the growth of Strain JF1754, and consisted of 1% N-2 amine type A, 0.5% yeast extract, 0.5% NaCl, titrated to pH7 prior to autoclaving. "LB" was the same media supplemented with 0.2% dextrose. Minimal bacterial medium (M9) consisted of 7 g/l (anh) Na_2HPO_4 , 3 g/l (anh.) KH_2PO_4 , 1 g/l NH_3Cl , 1g/l NaCl (from a 10 X stock), 0.1 mM CaCl_2 , 1mM MgSO_4 , 0.2% glucose, 20 $\mu\text{g/ml}$ Thiamine, 50 $\mu\text{g/ml}$ each of thymine and uracil. Solid media contained 2% agar.

For large-scale (0.5 or 1 liter) plasmid preparations from JF1754, 1 g/l Bacto-Casamino acids was added, plus ampicillin at 0.1 mg/ml to select for the plasmid (M9-amp). For bacterial "mini-preps", cells were normally grown in LB plus 0.1 mg/ml ampicillin (LB-amp).

B.3. Plasmid constructions

B.3.1. Restriction and ligation of plasmid DNA. To move fragments of DNA from one vector to another, one of two procedures was followed. In the first procedure (cf. Figure 2b), the fragment of interest (the LEU2 gene on a Sall, XhoI fragment in pYF91) was excised from the plasmid by digestion with the restriction endonuclease and purified by electrolution of the fragment from an agarose gel (Maniatis, 1982). This fragment was mixed and ligated using T₄ DNA ligase with a second plasmid (pRS231) which had been cut once (with Sall) to generate ends cohesive to those of the fragment. The ligated DNA was transformed into E. coli, and the plasmid transformants selected (for the presence of the LEU2 and amp^r genes, in this case). With the second procedure, the fragment was not purified prior to mixing (cf. Figures 5a and 6), but the correct ligation product instead was screened for in E. coli JF1754 transformants obtained using the ligated mixture. In both cases, derivative plasmids were then isolated from the transformed JF1754 (see below), screened and verified by restriction endonuclease digestion, agarose gel electrophoresis of the digest and identification of predicted DNA bands (unpublished).

B.3.2. Transformation of plasmids into E. coli. A simplified version of the Mandel and Higa (1970) calcium chloride procedure was used to introduce plasmid DNA into E. coli strain JF1754. 0.5 ml of a fresh 2 ml overnight culture from a single JF1754 colony was inoculated into a 50 ml LB culture in a 200 ml flask. This culture was grown to an OD₆₀₀ of 0.16, then the flask was put in a slush bath to cool to 2°C. The cells were transferred to oak ridge tubes, spun at 6000 rpm (IEC 8 x 60 ml rotor) for 2' at 4°C, washed in 10 mM MgSO₄, and resuspended in 30 ml of 70

mM CaCl_2 . The cells were left to become competent for one hour on ice, re-centrifuged (same conditions) and resuspended in 2 ml of CaCl_2 . 0.2 ml aliquots of this suspension were added to (microfuge tubes (Eppendorfs), and up to 0.1 μg of ligated DNA was added in 10-25 μl of TE. This mix was allowed to stand on ice for at least one hour, then mixed with 0.2 ml of LB and plated onto LB-amp.

B.3.3. Plasmid isolation from E.coli The particular recipe for the rapid isolation of plasmid DNA from JF1754 was derived for that of Birnboim and Doly (1979). For isolating putative recombinant plasmids, a single transformed colony was picked from an LB-amp plate into 2 ml of liquid LB-amp and grown to saturation (<15h) with shaking. 1.5 ml of this culture was centrifuged for 5 seconds in an Eppendorf centrifuge (12000 rpm), and the pellet was thoroughly resuspended in 0.2 ml of pre-lysis solution (25mM Tris-Cl, pH8; 10mM EDTA; 50 mM dextrose and 5 mg/ml lysozyme, added before use) by vortexing. After 5 minutes' incubation at 30°C, 0.4 ml of a 0.2 N NaOH/1% SDS solution (freshly made, at room temperature) was added and the suspension gently mixed until clear (usually \approx 5 min.). At this time the Eppendorf tube was placed on ice and 0.3 ml of ice cold NaOAc (pH4.6) was added to precipitate cell debris and SDS. After 5 minutes the prep was centrifuged at 12000 rpm for 30 minutes at 4°C. The supernatant containing the DNA was removed to a clean Eppendorf tube at room temperature and precipitated with 0.6 ml of room temperature isopropanol (this appeared to yield cleaner preps than those precipitated with cold isopropanol) plus intermittent vortexing for 10 minutes, then centrifuged for 10 minutes at 12000 rpm. The prep was washed once with 50% isopropanol and once with ice cold 70% ethanol, dried and resuspended in 40 μl of TE (10mM Tris-Cl(pH8), 1mM EDTA)

buffer, then spun for 5 minutes to pellet residues. 5 μ l of the prep containing roughly 0.3 μ g of plasmid DNA was digested with the appropriate restriction endonuclease, and the prep was extracted with chloroform if difficulties were encountered with digestion. These "mini-preps" took about 3 hours to prepare, and were stable for up to one month. The cell pellets from the first centrifugation could also be stored at -80°C (E. Calmels, pers. comm.).

Large-scale isolations of plasmid DNA for biochemical manipulations were achieved by a scaled up version of the above procedure. Cells bearing a known plasmid from a 2 ml culture pregrown as described above were inoculated into 500 ml of M9-amp medium with Casamino acids, incubated with shaking overnight, spun down (6000 rpm, 4 min) and thoroughly resuspended in 6 ml of pre-lysis mix (using 10 ml pipettes). The cells were transferred to an oak ridge tube, vortexed intermittently over 5-10 minutes, lysed by the addition of 12 ml of NaOH/SDS, then placed on ice. 9 ml of ice-cold NaOAc was added, and after 5 minutes the oak ridge tube was centrifuged at top speed in a refrigerated IEC centrifuge at 4°C . The supernatant was transferred to a clean oak ridge tube, which was then "topped up" with room-temperature isopropanol. The rest of the procedure followed the mini-prep format.

The DNA was resuspended in TE by intermittent vortexing (over one hour), then 6.85 ml of the prep was transferred to clear 16 x 76 mm Beckman ultracentrifuge tubes containing 7.50 g of CsCl, and capped with titanium caps. Parafin was added to displace air in the tube, then 0.9 ml ethidium bromide from a 10 mg/ml stock was injected through the cap orifice with a 1 ml syringe. The cap was then sealed and the tube was weighed and balanced to 0.1 gm against another prep, then centrifuged at 37000 rpm (60.1 Ti rotor (Beckman) in a Beckman (model L8-70)

ultracentrifuge at 15°C for 60 hours. Once the tube vacuum was broken by removing the top orifice screw, the plasmid DNA, evident as the lower of two bands under UV light, was withdrawn into 8ml test tubes with a medium bore, 2 ml syringe. The ethidium was removed by extraction with salt-saturated isopropanol. The prep was then dialysed against TE to remove CsCl, removed into an Eppendorf tube, extracted twice with phenol:chloroform:isoamylalcohol (25:24:1) and four times with chloroform:isoamylalcohol (24:1). The purified prep was brought to 0.1 M NaCl, precipitated with 100% ethanol (-20°C), washed twice with 70% ethanol (-20°C), dried and resuspended in 100-500 µl of TE.

B.3.4. Transformation of plasmids into yeast. Once a particular plasmid containing the gene of interest had been constructed, one of two methods was employed to transfer the plasmid into yeast. Initially the spheroplasting procedure of McNeil *et al* was used. 40ml of cells growing in YEPD were harvested in the logarithmic (log) phase of growth at about 10^7 cells/ml, centrifuged at 3000 rpm in oak ridge tubes, washed once in 1M sorbitol, then resuspended in 4ml of Sorbitol plus 1% glucylase (Endo) to digest the cell wall. The cells were incubated at 30°C with gentle shaking for one hour or until lysable with 10% SDS, then centrifuged at 2500 rpm and washed twice with 30 ml of 1M sorbitol to remove the glucylase. The spheroplasts were washed with 20 ml of STC (1M sorbitol; 10mM Tris-Cl pH7.5; 10mM CaCl_2) and resuspended in 0.5 ml of STC. At this stage 100 µl of the spheroplast suspension was mixed with 1 µg of plasmid DNA, incubated for 15 minutes at 30°C, then mixed with 10 volumes of PTC (20% polyethylene glycol (PEG) 4000, 10 mM Tris-Cl pH7.5, 10mM CaCl_2). After a further 15 minutes' incubation, the PTC was removed by another low speed centrifugation and washed with STC.

1/20th of this suspension was mixed with 10ml of regenerating agar in pre-warmed test tubes and poured onto YNBD medium supplemented with histidine. Transformants to leucine prototrophy were recovered after 2-4 days incubation of the plates at 30°C. These strains were then tested for the presence of unselected markers present on the plasmid (eg. the introduced lacZ gene).

The LiCl method of Ito et al (1983) was also used. Cells were harvested in late log phase, washed in 10mM TE (pH8) and resuspended in 2ml of the same solution. An equal volume of 0.2 M LiCl was added and the mixture was shaken gently for one hour at 30°C. 0.1 ml aliquots were transferred to Eppendorf tubes, and up to 10 µg of DNA was added per tube. Following an additional 30 minute incubation at 30°C with gentle shaking, an equal volume of 70% PEG 4000 was added and immediately mixed in by vortexing. The suspension was left to stand at room temperature for an additional hour, heat shocked to 42°C for 5 minutes, centrifuged at 3000 rpm for 3 minutes, resuspended in liquid YNB and plated to selective medium.

B.4. Generation of synchronous yeast populations .

Yeast strain AH22 was synchronized by α -factor arrest, based on the procedure described by Hereford et al (1981). Two days before the synchrony experiment, AH22 derivative cells of a mating type stored on YEPD plates were inoculated into 2 ml of selective medium, and allowed to grow for 24 hours into stationary phase. At the same time, cells from the α -factor producing yeast strain S288C were inoculated into 100 or 200 ml of YNBD (6.7 g/l Difco YNB plus 2% glucose in one or two liter flasks) culture, and grown to stationary phase for 40-48 hours with vigorous shaking. The following evening 20 to 100 µl of AH22 culture

was transferred to 100-200 ml of synchrony medium, which was then grown overnight to a density of $> 2 \times 10^6$ per ml. On the third day just prior to the experiment, the S288C culture was spun down (3000 rpm), then the α -factor-containing supernatants were passed through a Nalgene 0.2 μ m filter and saved. When the AH22 derivative achieved the appropriate density the cells were pelleted in oak ridge tubes and quickly resuspended in 100-200 ml of "0.1%" arresting medium (0.1% YEP, 100 μ g/ml histidine, 4% dextrose), in a two-liter flask. An equal volume of filtered α -factor supernatant was then added to the culture, and the melange was incubated for roughly 90 minutes until the cells were $> 90\%$ unbudded. At this point the α -factor was washed out of the culture by filtering or by centrifugation, and the cells, now synchronized at the α -factor block, were resuspended in 100-400 ml of synchrony medium. 1-40 ml samples of culture were then taken for assays, while 0.5 ml samples were mixed with 0.5 ml 3% formaldehyde in synchrony medium, at intervals of 5-10 minutes. These samples were used for cell counts and for culture OD measurements.

The approximate amount of α -factor (Sigma) used to arrest 25 ml of AH22 log phase cells at 5×10^6 cells/ml under the conditions described above was 50 μ g (2 μ g/ml); the S288C-produced α -factor medium used in these experiments was assumed to be 30 μ g/l (Manney et al. 1981).

B.5. Harvesting and storage of cell samples from yeast cultures.

1 ml to 10 ml samples of cells in liquid 0.1 % YNB medium were centrifuged at 4°C for 2 minutes (3500 rpm). The supernatant was discarded and the pellets were either assayed immediately or frozen in liquid nitrogen [N₂(l)] and stored at -80°C. These samples were used to

determine enzyme activities. Samples for RNA were larger; 10-35 ml of culture was added to tubes containing sodium azide and 10 ml of crushed distilled-water ice, pelleted and frozen.

B.6. β -galactosidase (β gal) assays.

β -galactosidase activity was detected as described by Miller (1973) and by Casadaban et al. (1980) with the following modifications, in two separate procedures. In the first procedure, unfrozen samples were resuspended in 0.7 ml of Z₁ buffer (Miller, 1972) containing 4 mg/ml o-nitrophenyl- β -D-galactopyranoside (ONPG; Sigma) and then 25 μ l each of chloroform and 0.1 % SDS were added. This procedure also worked for frozen cells. (Note that this relatively high concentration of ONPG in the reaction mix (1.14 mg/ml was used by Miller), sometimes results in a precipitate forming, particularly if the ratio of reaction buffer to carbonate stop solution is reduced, or if the Brij 35 is exposed to light for more than a few days; data not shown).

In the second procedure, frozen pelleted samples were thawed in 0.77 ml Z buffer containing β -mercaptoethanol and 4 mg/ml ONPG. (Miller, 1972), and 3 % Brij-35 (Fisher). This assay solution could be refrigerated, if tightly sealed, for up to two weeks. The frozen samples were resuspended by vortexing vigorously for 15 seconds and then incubated at 28 C until the suspension turned slightly yellow. The reaction was stopped by adding 0.5 ml of 1 M sodium carbonate and chilling the samples on ice. Failure to chill the samples following the addition of carbonate resulted in increasing ODs from the blank using either procedure. Cell debris was removed by centrifugation, after which the OD (420nm) was measured. Except where noted, this "N₂(1)/Brij" permeabilization was routinely used for β gal assays in this study. The

formula used to convert OD to β -galactosidase (β gal) activity, based on that of Schleif and Wensink (1981), was

$$\text{Units of Activity} = \text{pmol/min.ml} = \frac{\text{OD}_{420} \times 1000}{0.0049 \times V \times t}$$

where 0.0049 = OD/nmol of ONP product per ml of assay; V = the original volume (ml) of culture in the assay, and t = the time of assay in minutes. Note that the 4 mg/ml initial concentration of ONPG in the assay was 3.5 times more concentrated than that used by Miller (1972).

B.7. RNA preparation and glyoxal gel electrophoresis.

RNA was isolated as described in Storms *et al* (1984; see also Figure 2). The cells were resuspended in 1 ml of breaking buffer (0.5 M NaCl, 0.2M Tris-HCl pH7.5, 0.01M EDTA), transferred to Eppendorf tubes and repelleted. 100 μ l breaking buffer was added to the pellet, which was resuspended and transferred to screw-cap Eppendorf tubes containing glass beads, phenol:chloroform:isoamyl alcohol (25:24:1), and 0.1% SDS (final W/V). The phenol contained 0.2% β -mercaptoethanol, and 8-hydroxyquinoline was added to the solvent mix just prior to use. Each Eppendorf tube was vortexed for 5 minutes in one minute bursts, then centrifuged at 12,000 rpm for 15 minutes. The upper aqueous phase was transferred to a fresh screw-cap Eppendorf tube which contained 200 μ l chloroform; isoamyl alcohol (24:1). The phenol phase was back extracted with 100 μ l of breaking buffer, and the upper phase added to the fresh Eppendorfs. The combined aqueous phases were extracted with the chloroform by vortexing for 15 seconds, and centrifuged at 12K rpm for

15 seconds, the chloroform was discarded, fresh chloroform was added and the step was repeated 2-3 times. The aqueous phase was then precipitated in 2.5 V of absolute ethanol, washed with 75% ethanol and resuspended in 25 μ l of water.

All aqueous solutions used after the solvent extraction were pre-treated overnight in a fume hood with 0.1% diethylpyrocarbonate and then autoclaved twice. Following resuspension, 5 μ l of each RNA prep was used to measure the RNA concentrations of the different samples, which were then adjusted to \approx 5 μ g/ μ l. Prior to use, 15-20 μ g of this RNA in 4-5 μ l water was mixed with 16 μ l of DMSO and 4 μ l of 0.1M NaH_2PO_4 , pH7.5 pre-incubated for ten minutes at 50°C. 16 μ l of glyoxal was then added, and incubation continued for 50 minutes. 2 μ l of loading buffer (50% glycerol, 0.01M NaH_2PO_4 pH7.5, 0.4 % bromophenyl blue) was added, and the samples were loaded onto duplicate 1% agarose slab gels and electrophoresed in 0.01M NaH_2PO_4 buffer, pH6.5 at 90 volts for roughly three hours. The bottom gel was stained with EtBr to detect loading errors as well as rRNA molecular weight markers (4.4 and 1.6 Kb, respectively).

B.8. RNA "Northern" blotting and probe hybridization.

RNA was blotted from the unstained gel to nitrocellulose (Schleicher and Schuell) or Biodyne (Pall) membranes, using filtered 20X SSC and a membrane pre-soaked in water, then 20X SSC. After transfer the gel was stained with EtBr to detect untransferred regions. The membrane was baked for 1 hour at 80°C then stored at -20°C. Filters were hybridized to radioactive probes as described by Thomas (1980), and exposed to Kodak Curex Xray film. α - ^{32}P -labeled DNA for probes was prepared by nick-translation with E. coli DNA polymerase I (Maniatis, 1982) to a

specific activity of more than 10^7 dpm/ μ g. The probes used are given in the particular Figure legends. In order to reprobe a blot, previous probe DNA was stripped from the filter by incubation in water at 95°C for 5 minutes, or as described in the legend to Figure 2 (Thomas, 1983).

C. RESULTS

C.1. IMP1 is a CCR gene.

In the initial stage of this study, the regulation of yeast TS was shown to be cell cycle dependent (Figures 1 and 2; from Storms *et al.*, 1984). In cells synchronized by centrifugal elutriation (*ibid*; not shown here) or by the α -factor technique (Figure 1), TS activity (as measured by a $6\text{-}^3\text{H-dUMP} \rightarrow ^3\text{H}_2\text{O} + \text{dTMP}$ "tritium release" assay; Bisson and Thorner, 1981) peaked during S phase. The end of S phase corresponds to the peak in proportion of newly budded cells, or to the lowest proportion of unbudded cells, in strain AH22. The ability of TS to irreversibly bind the dUMP analogue FdUMP, was exploited to show that quantities of active TS, as well as catalysis (activity), peak at this time (Figure 2, center).

Periodic increases of TS were shown to be determined primarily at the level of the transcript (Figure 2, bottom panel). Levels of RNA per mg total RNA peak at the beginning of S phase in the first generation. A smaller peak is observed in the second generation, corresponding with the onset of the subsequent S phase, which actually starts prior to cytokinesis in this strain. The possible significance of this profile is elaborated in the discussion.

As a result of this preliminary work, it is evident that IMP1 is a "CCR" gene, as defined in the Introduction. I then set out to determine whether sequences in the 400 bp flanking region immediately adjacent the TS coding region of the cloned IMP1 gene (Taylor *et al.*, 1982) were responsible for its cell cycle dependent regulation.

C.2. Strategy for Studying the IMP1 5' Regulatory Region.

Once it was known that the yeast IMP1 gene was periodically

Figure 1. DNA content, acid phosphatase activity, and thymidylate synthase (TS) activity in α -factor-synchronized cultures (from Storms *et al.*, 1984). Cells were synchronized with α -factor, and samples were removed at 10 minute intervals. All the data are from a single synchronous culture. (a) Acid phosphatase levels as determined colorimetrically in permeabilized cells and DNA content as determined by a diphenylamine assay (*ibid*). (b) TS activity, proportion of cells without buds, and cells with small buds. TS activity per ml of synchronous culture was followed in whole cells permeabilized with Brij 35 by using the tritium release assay (*ibid*). Cells in which buds were less than one-half the diameter of the mother cells were defined as cells with small buds. For each time point, at least 300 cells were scored microscopically for bud morphology. Cell samples for determining bud morphology were taken at 10 minute intervals starting at $t=0$ min, and samples for phosphatase activity, DNA content and thymidylate synthase activity were harvested simultaneously at $t=0$ and then at ten minute intervals after $t=13$ min. The profile of TS activity versus time seen here was observed in each of three additional experiments (data not shown). Student's t test was used to compare TS activities at the peak from all four experiments, with the activities found at the first and the second time points immediately after the peak (ie. a comparison of $t=53$ min and $t=63$ min for the above experiment). These paired comparisons showed that TS activity was significantly reduced at both the first and the second time points after the peak, with $P,0.005$ and $P,0.01$, respectively.

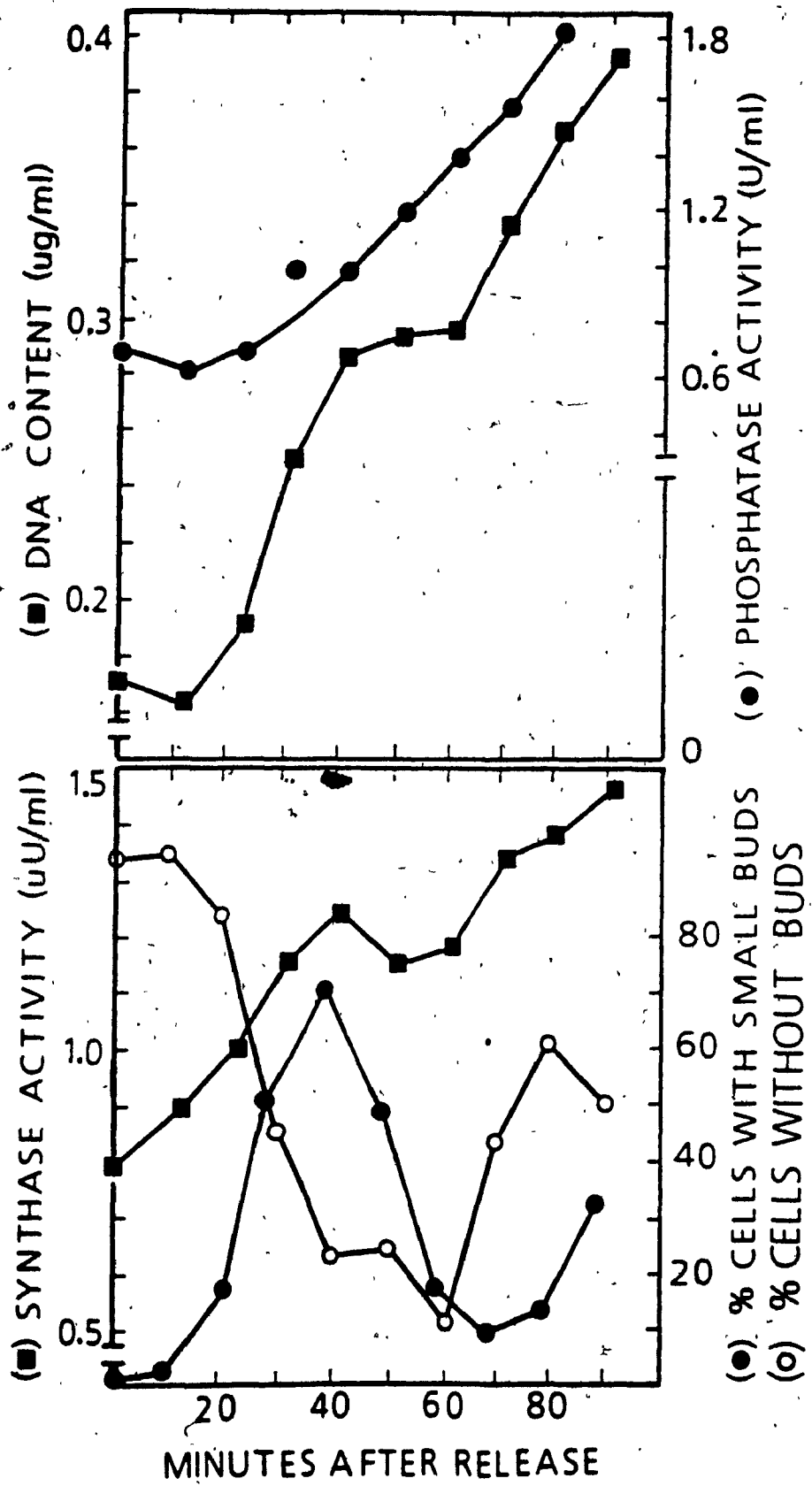
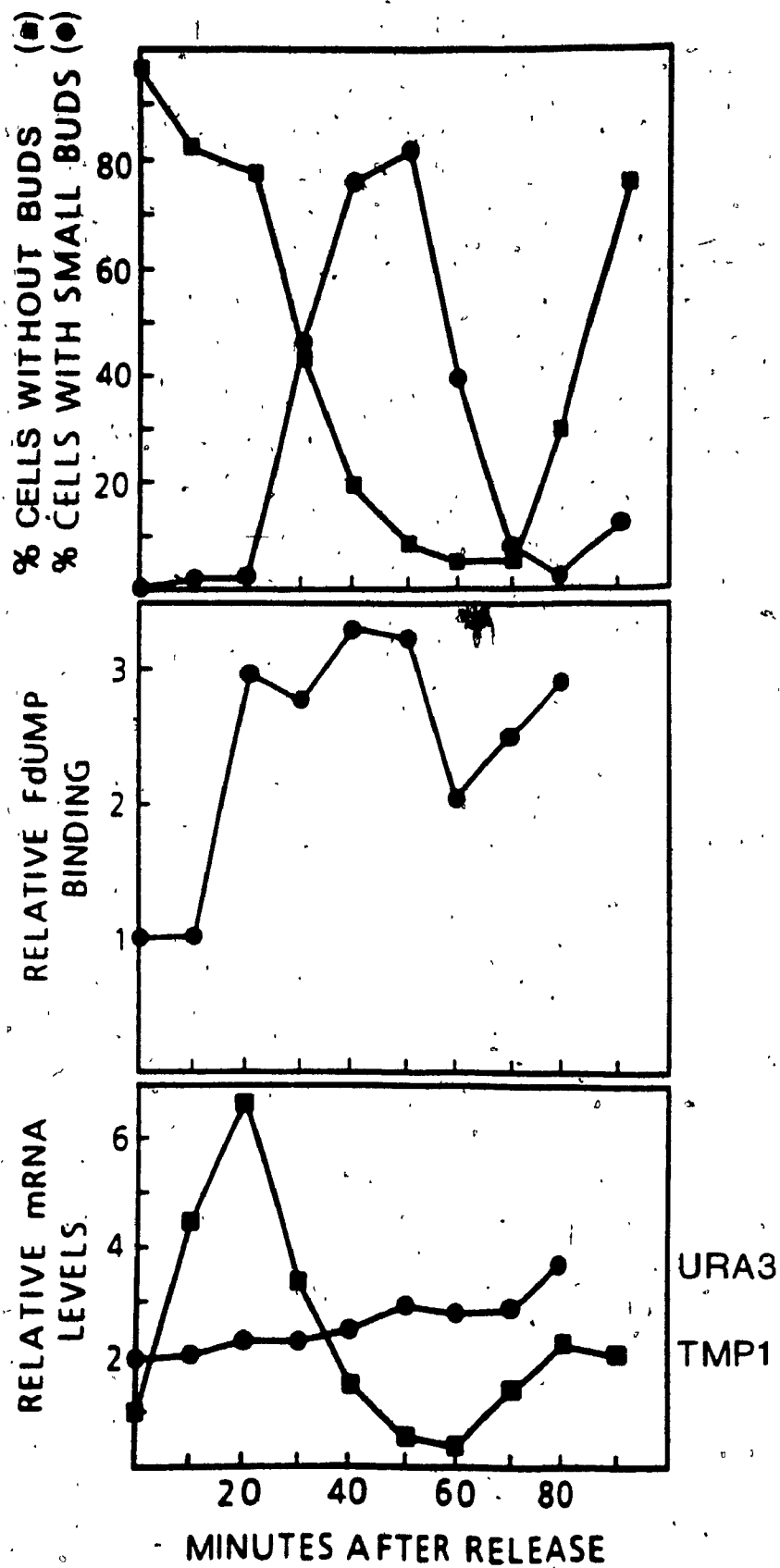


Figure 2. Levels of FdUMP binding and IMP1 transcript during synchronous growth after release from α -factor arrest. (a) Percentage of cells without buds (■) and with small buds (●) at different times after release. (b) Levels of FdUMP-binding activity quantitated as described by Storms et al (1984). The points represent an average of two separate fluorograms. (c) Quantitation of TS transcript. RNA was extracted from cells removed at the indicated times. The RNA was analysed on a Northern gel, using the labelled EcoRI to HindIII fragment of the S. cerevisiae IMP1 gene (see Figure 3) to identify the IMP1 transcript (the autoradiogram is shown in Storms et al, 1984). Only a single RNA species was detected, 1.1 Kb in length. Relative levels of IMP1 RNA at various times during synchronous growth were quantitated by densitometry (■). The hybridized DNA was eluted by washing the DNA in 0.1 x wash buffer (Thomas, 1980: 1 x wash buffer contains 50mM Tris-HCl, pH8.0; 2 mM EDTA; 0.5% sodium pyrophosphate; and 0.02% bovine serum albumin, Ficoll and polyvinyl pyrrolidone) for two hours at 65°C, and then the blot was reprobed with the 0.9 Kb PstI to SmaI fragment of plasmid Ylp5, which contains the yeast URA3 gene (●). Whereas the data in (a) and (c) represent the same synchrony, the FdUMP binding data in (b) are from an independent experiment. The timing of the two synchronies in these two cases, however, was indistinguishable.



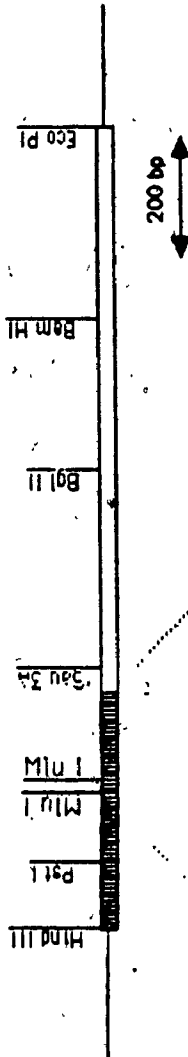
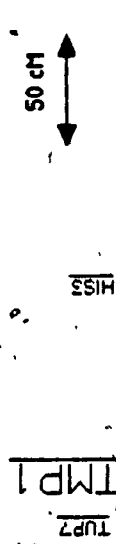
expressed, I wanted to determine where the information responsible for this regulation was located. In order to identify and localize the regulatory sequences, I chose to construct a gene fusion which placed the E. coli β -galactosidase gene, lacZ, under the transcriptional and translational control of the regulatory region from the yeast IMP1 gene. If the regulation of β -galactosidase (β gal) activity expressed from such a fusion mimicked the cell cycle regulation of thymidylate synthase, then the regulatory information could be further localized by deletion analysis.

C.2.1. Construction of plasmids bearing IMP1'-'lacZ fusion genes.

The proposed study of IMP1 regulation via IMP1'-'lacZ gene fusions required a suitable expression vector, that is, a plasmid containing the fusion gene which could be selected and could replicate autonomously in yeast and in E. coli. The construction of these IMP1'-'lacZ gene fusions began with the previously cloned IMP1 gene on plasmid pTL1 (Taylor et al., 1982). The chromosomal location of the IMP1 gene, and a partial restriction map of this region are given in Figures 3A and B; the 5' DNA sequence up to the vicinity of the CGCG 5' repeats noted in the Introduction is shown in Figure 3C. Using this plasmid as well as pYF91 and pMC931, the first expression vector pRS264 was constructed as shown in Figure 4. The first step was to insert the BamHI to BglII fragment containing the 'lacZYA' information from pMC931 into the BamHI site of the yeast shuttle plasmid pYF91, generating pRS58 (Figure 4A). The second step (Figure 4B) was to replace the small BamHI to HindIII fragment upstream from 'lacZYA' on pRS58 with the 0.97 kb HindIII to BamHI fragment of IMP1 on pTL1 to generate plasmid pRS231. The final step in the construction of pRS264 was to insert the yeast LEU2

Figure 3. Chromosomal location (top), restriction map (center) and immediate 5' sequence (bottom) of the yeast IMP1 gene. Chromosomal distances are in centimorgans (cM; % meiotic recombination between genes), while restriction map units are in base pairs (bp). The structural gene region is represented by an open box (the coding region ends 18 bp after the EcoRI site). The vertically hatched box indicates the 5' regulatory region studied here. The HindIII-Sau3A segment was used to generate IMP1'-'lacZ gene fusions. Shown at the bottom is the downstream portion of the HindIII-Sau3A sequence of the 5' regulatory region (see Figure 1.3: for a complete IMP1 sequence map). Putative regulatory sequences include a region between the two MluI cut sites which is implicated in the cell cycle regulation of IMP1, TATA box sequences and the start of the coding region as predicted from the protein sequence (Bisson and Thorner, 1981), are underlined.

Chromosome XV



C

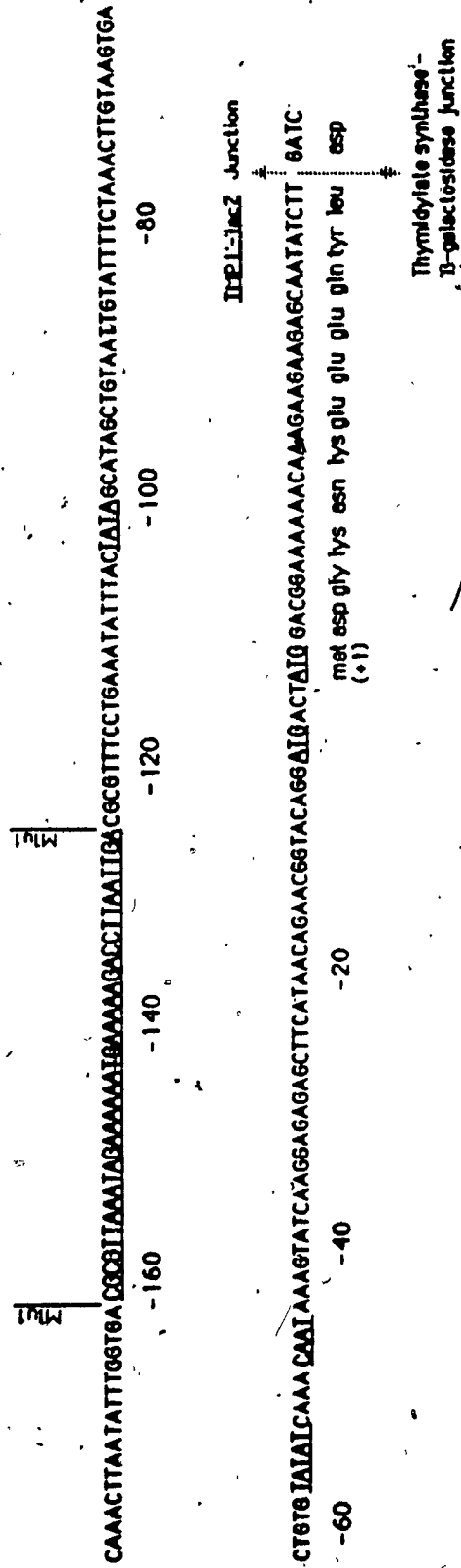
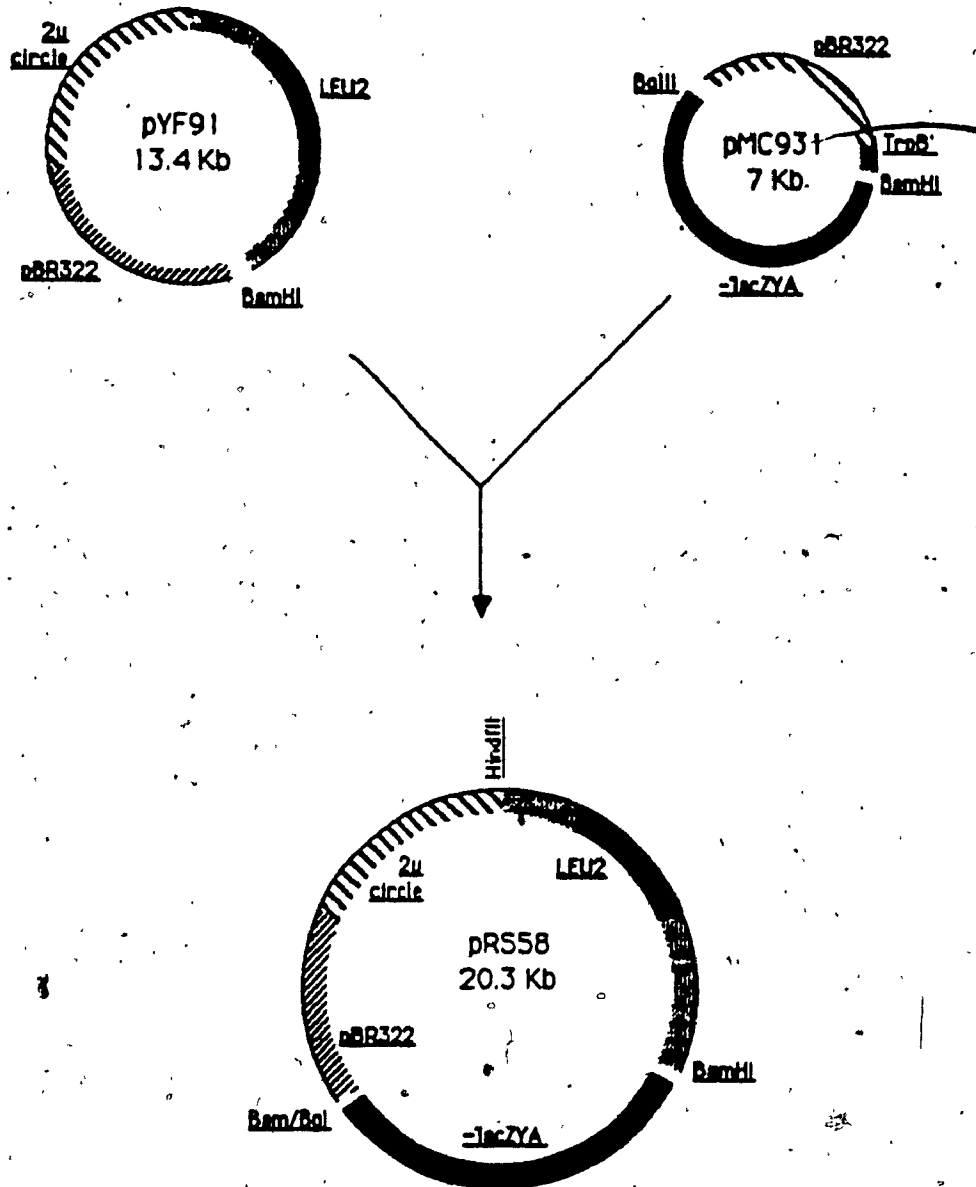


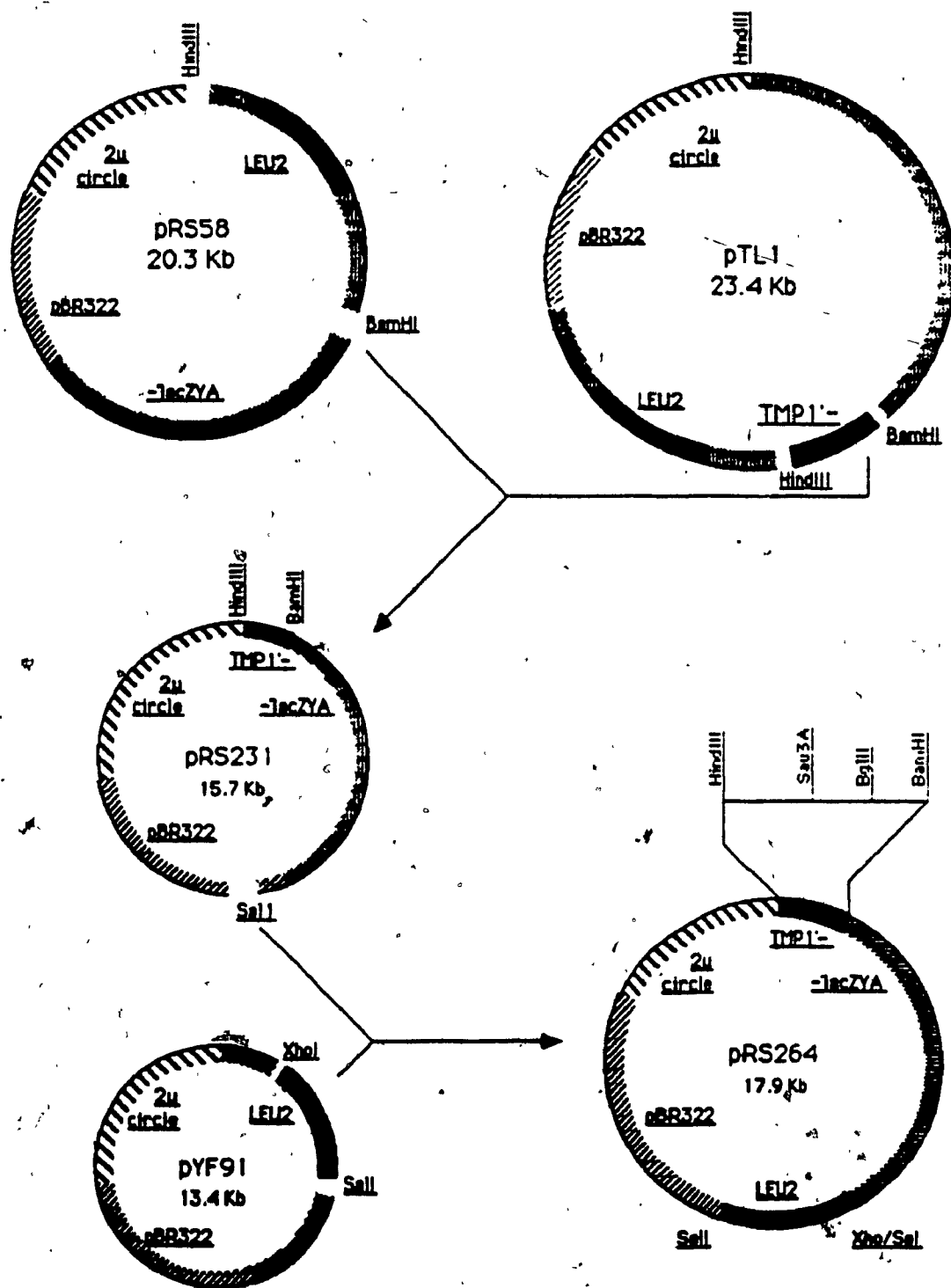
Figure 4. Preliminary plasmid constructions (Storms, Taylor and Ord, unpublished). For simplicity, only restriction sites used in the particular construction are indicated.

- A. Introduction of lacZ into pYF91. The pRS58 construction provided the *E. coli* pBR322 sequences (including an origin for replication, and the amp^r gene for selecting the plasmid, in *E. coli*), yeast 2 μ -plasmid sequences (including an origin for replication of the plasmid in yeast), the yeast LEU2 gene (for selecting the plasmid in yeast) and lacZ sequences (whose 5' end lies at the BamHI cut site).
- B. Construction of the out-of-frame IMP1'-lacZ fusion plasmid pRS264. The HindIII - BamHI yeast IMP1' sequences originally cloned in pTL1 were introduced into the pRS58 HindIII - BamHI LEU2 fragment, generating pRS231. A smaller, XhoI - Sall LEU2 fragment was reintroduced into pRS231 to generate pRS264. An in-frame version of pRS264 (pRS264i, constructed by M. Greenwood) was subsequently generated by BamHI digestion of pRS264 followed by S1 nuclease removal of the staggered end and religation.

A



B



gene contained on a 1.7 Kb fragment flanked by Sall and XhoI sites into the unique Sall site of pRS231. These manipulations resulted in the construction of pRS264 (Figure 4B). The resulting IMP1'-'lacZ fusion gene contained on pRS264 places lacZ under the transcriptional and translational control of the IMP1 regulatory region. This plasmid is capable of autonomous replication in yeast, due to the presence of the yeast 2 μ -circle replication origin (ARS).

Plasmid pRS264 expressed very low levels of β -galactosidase in yeast (see below). This result was consistent with IMP1 sequences (Taylor, 1986) and lacZ sequences (Casadaban et al, 1983) at the fusion junction, which predicted that translation originating at the IMP1 START codon would read into the lacZ coding region in the wrong translational reading frame (out-of frame translation), and so produce a truncated, inactive product.

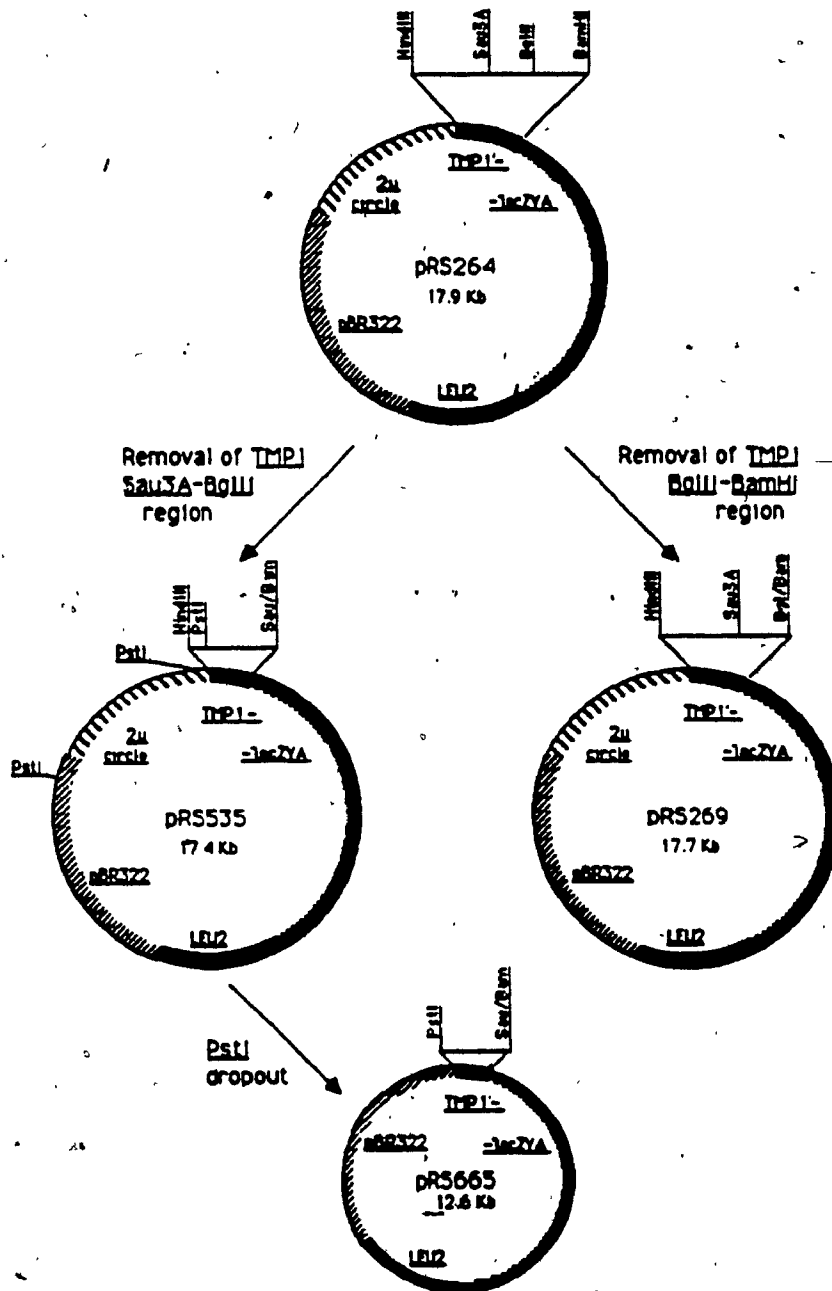
Three derivatives of pRS264 were constructed as shown in Figures 5A, and their IMP1'-'lacZ fusion genes are shown in Figure 5B. All three derivatives should result in the in-frame translation of lacZ (as predicted from the IMP1 DNA sequence) in yeast. The first plasmid, pRS269, was constructed by removing the information between the unique BamHI and BglII sites in pRS264. This was accomplished by digestion of pRS264 with these restriction endonucleases followed by circularization and ligation of the larger BamHI - BglII fragment. A smaller derivative of pRS535 also shown in Figure 5A, pRS665 was constructed by PstI restriction and religation of the largest pRS535 fragment (see below).

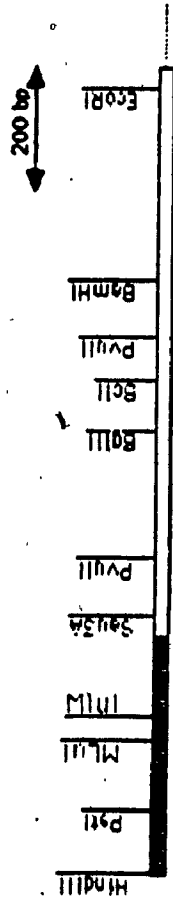
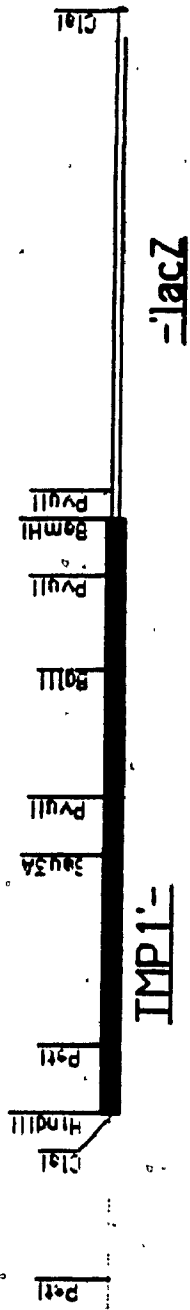
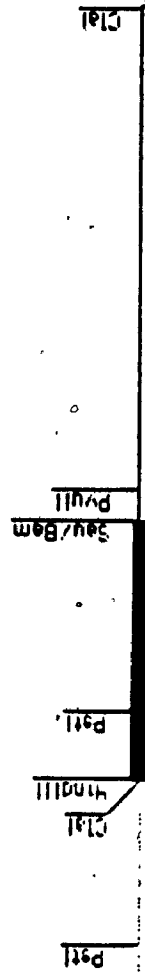
C.2.1.a. The autonomously replicating plasmid pRS535.

If a fusion gene's expression reflects IMP1 expression, then the periodic accumulation of β -galactosidase activity should reflect periodic fluctuations in its coding mRNA. Because β gal assays are easy to perform,

Figure 5. Construction and mapping of IMP1'-'lacZ expression vectors with intact 5' ends.

- A. Construction of the plasmids pRS269, pRS535 and pRS665. pRS269 was derived by cutting pRS264 with BamHI and BalII, and religating, while pRS535 was constructed by inserting the IMP1 HindIII to Sau3A fragment isolated from pRS479 into the pRS264 HindIII to BamHI site (essentially as shown in Figure 9). pRS269 and pRS535 are capable of autonomous replication in yeast since these retain the yeast 2 μ -plasmid replication origin. In contrast, pRS665, constructed by PstI digestion of pRS535, is deleted for all 2 μ -plasmid DNA, as well as for part of the pBR322 amp^r gene, and for the HindIII to PstI region of IMP1. As a result, pRS665 must integrate into yeast DNA to be propagated. The pRS269 plasmid contains sequences between the Sau3A and BalII cut sites which are required to generate an unstable fusion protein (Greenwood et al, 1986). The pRS535 and pRS665 plasmids do not contain these sequences, and produce a stable Bgal activity in yeast (see text).
- B. Restriction maps of the original IMP1'-'lacZ constructions compared with the cloned IMP1 gene (top). Shown are the cut sites normally used to generate and identify derived fusions. A 29 bp EcoRI - HindIII pBR322 sequence remains at the junction of the boxed, dark IMP1 region and yeast 2 μ DNA. The PstI site in yeast 2 μ DNA is used as a reference. Note that the lacZ gene continues past the ClaI site.



B**pTL1****pRS264****pRS269****pRS535**

the fusion gene's expression can be followed by a single enzyme assay

However, the first in-frame fusion constructed, pRS269, expresses a β gal activity which is unstable in yeast (Greenwood, 1986). A shorter TMPI'-lacZ fusion, pRS535 (Figures 5 and 6), was constructed to minimize the thymidylate synthase (TS) portion of the fusion gene product. This plasmid encoded a stable β gal activity in yeast. Hence, the unstable activity from pRS269 was associated with the TS portion of the protein fusion from that plasmid. In contrast, pRS535, the main plasmid used in this study, its derivatives, and the pYT760-RYP3 and pL6669-2 control plasmids (see below) were all found to encode stable β gal activities in yeast (Greenwood, 1986 and see below).

pRS535 (Figure 5A and Figure 6) was constructed by replacing the TMPI information between the HindIII and BamHI sites on pRS264 with the smaller HindIII-Sau3A portion of TMPI shown in Figure 3. This was accomplished by mixing and ligating a Sau3A-digested, TMPI HindIII-EcoRI fragment (isolated from the pTL1 derivative pRS479; Figure 9) with a separate HindIII, BamHI digest of pRS264. Since the restriction endonucleases BamHI and Sau3A generate identical cohesive ends, this manipulation results in the fusing of the shortest HindIII-Sau3A TMPI fragment (-430 to +39; Figure 3) to the large pRS264 HindIII-BamHI fragment. Of the plasmids recovered after transforming JF1754 with the ligated mixture, pRS535 gave the correct restriction pattern (Figure 7A). The plasmid was thoroughly mapped with other restriction enzymes, and the correct TMPI'-lacZ junction was subsequently confirmed by DNA sequencing (E. Calmels, pers. comm.). When transformed into yeast, the behavior of the TMPI'-lacZ fusion contained on pRS535 conformed to that predicted for a periodically expressed gene (see below). This plasmid, shown in Figure 6, was

- Figure 6. Circular and linear maps of the plasmid pRS535, aligned to the HindIII restriction site. Unique cut sites are given on the top linear map, while enzymes which cut several times are given below. Other plasmids used for this study contained the same structure, except for the region 5' to 'lacZ' as noted in the text.

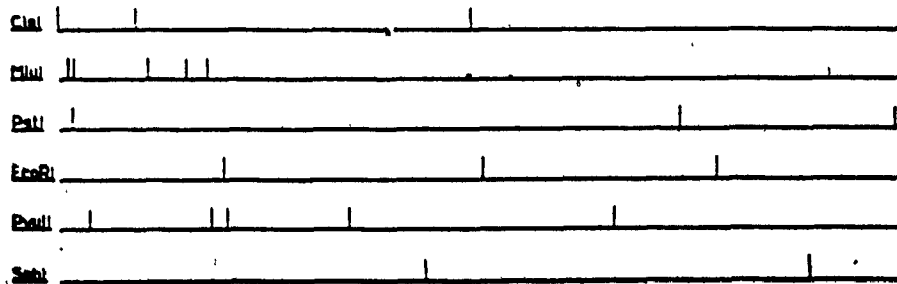
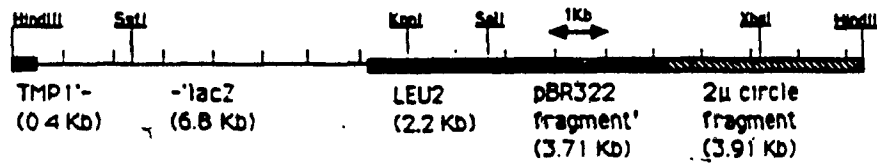
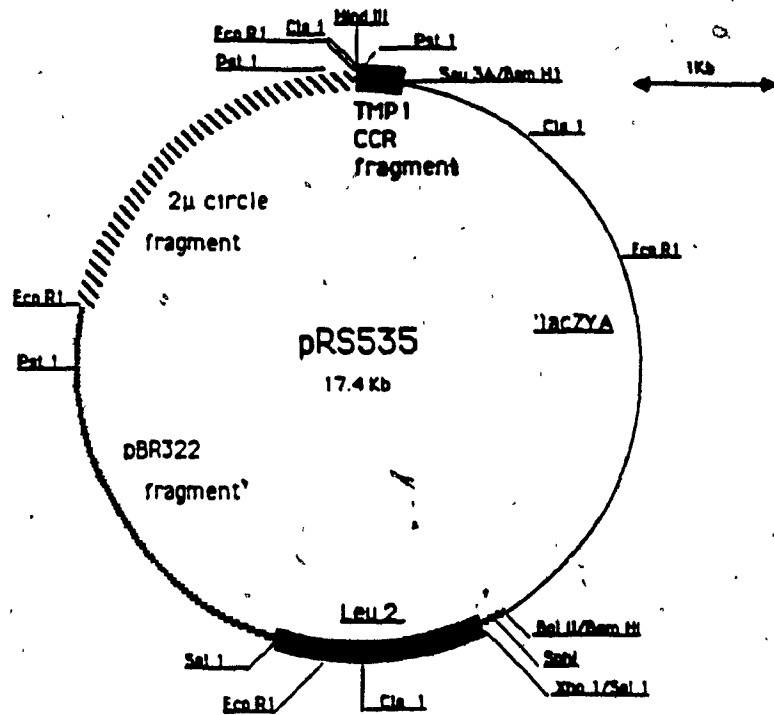
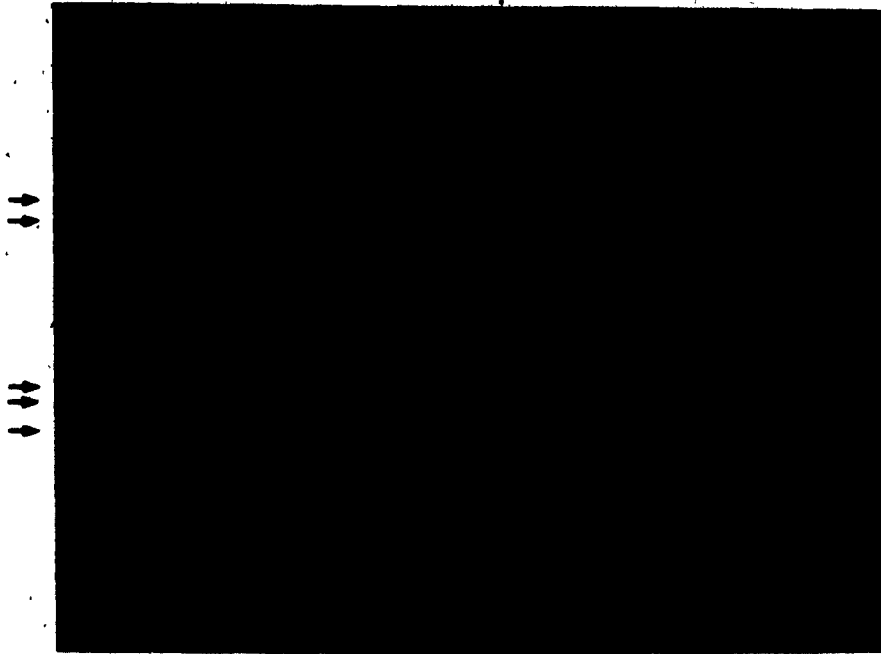
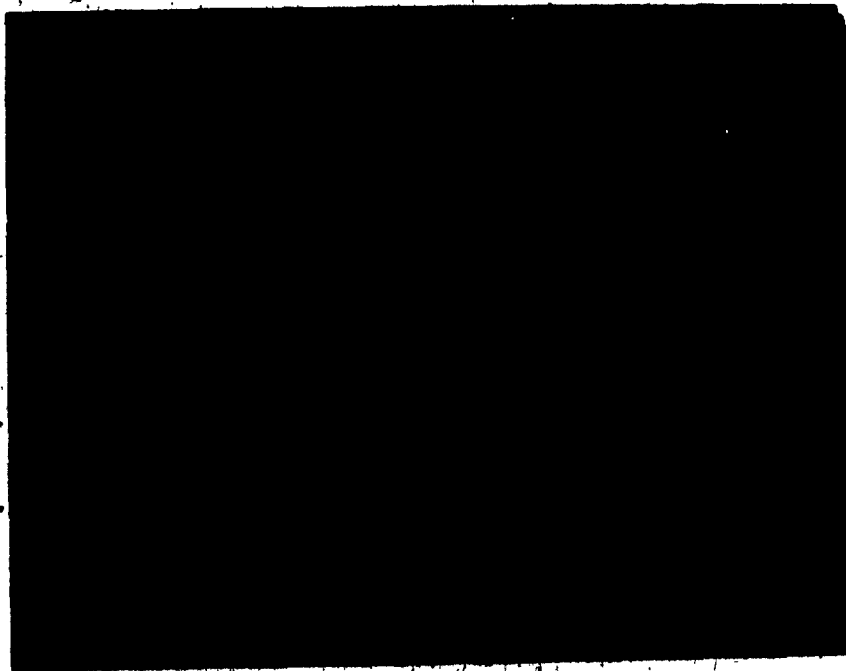


Figure 7. Typical restriction digests used for the characterization of plasmids constructed for this study.

- A. ClaI digests of pRS535 (lane 1), pRS264 (lane 3) and pRS269 (lane 4). From the top (arrows), fragments generated are, approximately, 9 kb, 7 Kb, then 2 Kb for pRS264, 1.8 Kb for pRS269 and 1.5 Kb for pRS535. Lanes 2 and 5 contain fusion plasmids pRS536 and 537, respectively, which were not studied extensively here. See Figure 6 for the predicted sizes of fragments. The additional faint bands observed between the 7 Kb band and the low MW bands were caused by "partial digests" of methylated ClaI sites present in the plasmids.
- B. PstI/ClaI digests of pRS535 (lane 1), pRS676 (lane 2) and pRS665 (lane 3). See Figure 6 for separate PstI and ClaI distances. From the top, common fragments generated in pRS535 are the 7 Kb ClaI fragment, two 4.4 Kb fragments and a 1.4 Kb PstI-ClaI fragment containing the IMP1 sequences (top arrow). In the pRS535 lane, the 0.25 Kb 2 μ -circle PstI-ClaI fragment is evident (bottom arrow), but was missing and subsequently confirmed to be absent from pRS676. The 0.1 Kb IMP1 ClaI-PstI 5' fragment was too small to be detected here. The other lanes contain digests of plasmids not studied here.

**A**

1 2 3 4 5

**B**

1 2 3

therefore considered the "wild-type", cell cycle regulated fusion gene.

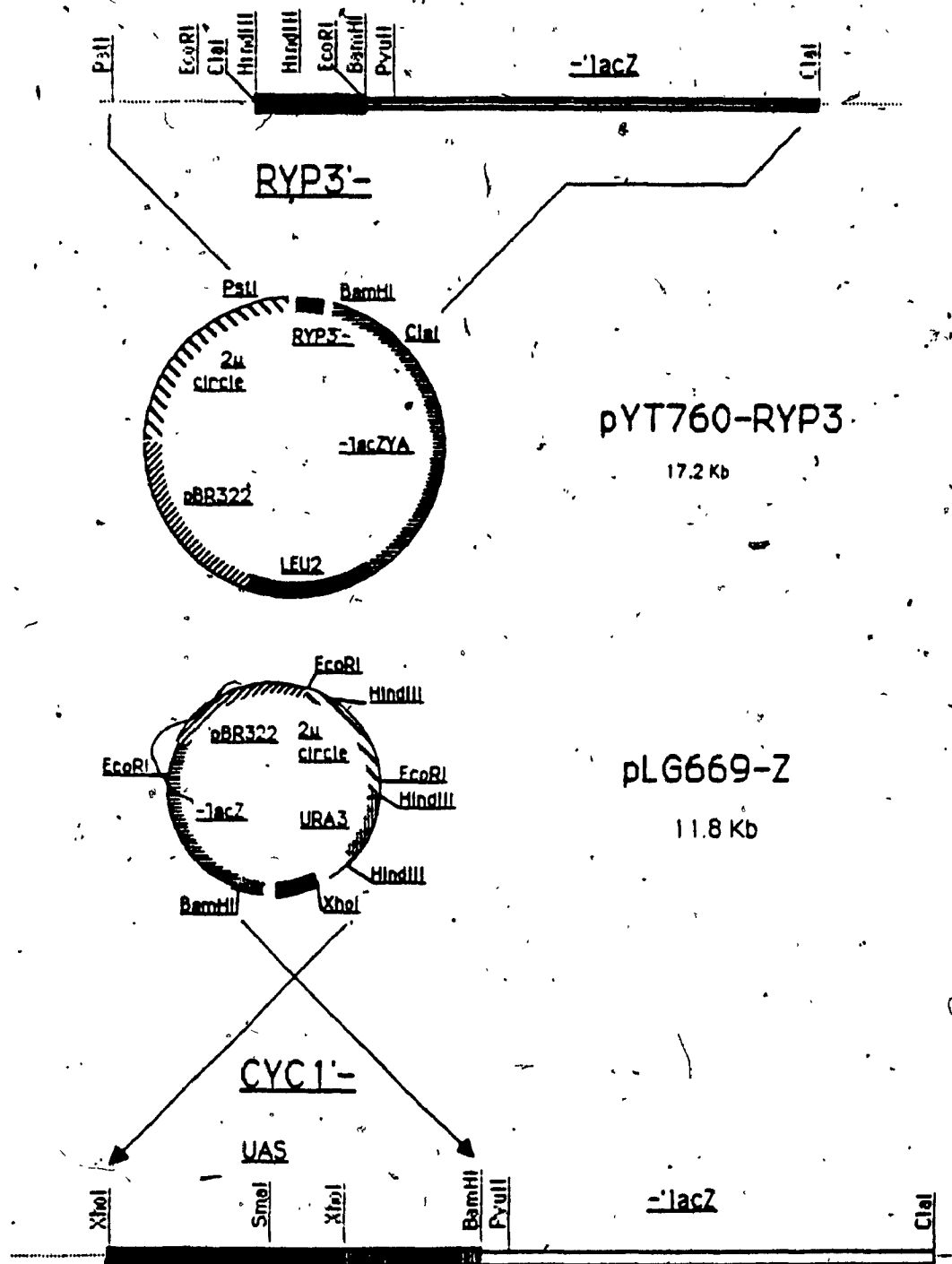
The ClaI restriction digest patterns of the initial plasmids constructed for this study are given in Figure 7A. Lane 1 shows one of four potential pRS535-type isolates, lane 3 shows pRS264 and lane 4 contains pRS269 (lanes 2 and 5 contained isolates later shown to contain longer inserts). The smallest ClaI band consists of the IMP1 fragment plus the 5' 'lacZ sequences (Figure 6). The middle band consists of a 6.8 Kb fragment containing the rest of the lacZYA sequences and part of LEU2, while the top band contains the rest of LEU2, 2 μ -circle and pBR322 sequences on a 9 Kb fragment. ClaI was generally used for preliminary mapping studies since changes in the smaller fragment, which contained the IMP1 regulatory information could be easily detected.

C.2.1.b. The Integrating Plasmid pRS665.

The periodic behavior (see below) of the IMP1-'lacZ fusion gene product allowed the fusion to be used to answer the following questions. First, is the periodic expression associated with IMP1 dependent upon the presence of an associated ARS which confers the ability to replicate autonomously (Osley and Hereford, 1982)? Second, does the expression of IMP1 depend on its genomic location? To address these questions, I constructed plasmid pRS665 (Figure 5A), which lacks the yeast 2 μ -circle ARS present on pRS535. This was accomplished by PstI digestion of pRS535 followed by ligation. PstI digested plasmids from a series of E. coli LEU⁺ transformants were screened by agarose gel electrophoresis to identify the appropriate plasmid. The plasmid was verified by endonuclease mapping (Figure 7B and L. Lee, pers. comm.).

Since pRS665 is missing the 2 μ -ARS, if transformation of yeast is to occur, the plasmid should integrate into a genomic site having homology with plasmid DNA (Orr-Weaver et al., 1981).

Figure 8. Circular maps of the promoter'-'lacZ plasmids used as controls for non-periodically regulated Bgal expression in yeast, pYT760-RYP3 and pL0669-2 (scale 1cm/kb), and linear maps (scale 1cm/100bp) of their promoter'-'lacZ fusion junctions. The first plasmid contains an unknown promoting activity 5' to 'lacZ, while pL0669-2 contains the CYC1 5' regulatory region. Both regulatory regions contain an ATG in-frame with 'lacZ DNA near the promoter'-'lacZ junction, separated from the 'lacZ junction sequences by less than ten bp. The region from each promoter with known Bgal promoting activity is indicated by a black box on the linear maps.



C.2.1.c. "Control" Plasmids.

Two other autonomously replicating plasmids, pYT760-RYP3 and pLG669-Z (Figure 8 & Table 2; gifts from D. Thomas and L. Guarente, respectively), were used as controls for non-periodic B-galactosidase expression. Plasmid pYT760-RYP3 contains an unknown yeast promoter sequence (RYP3) fused to lacZ instead of IMP1 information, but is otherwise identical to pRS264 and pRS269, while pLG669-Z contains the UAS, mRNA start sites and translational start sites of the yeast CYC1 gene fused to lacZ (Guarente and Ptashne, 1981) in a different plasmid.

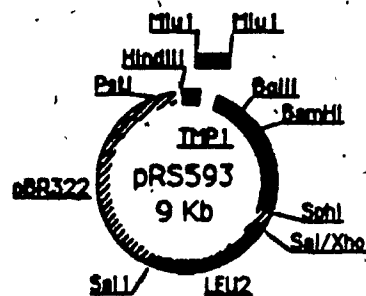
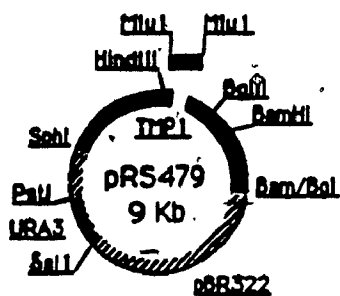
C.2.2. "pRS535" derivatives with altered IMP1 5' regions.

C.2.2.a. IMP1 Regulatory Region Rearrangements.

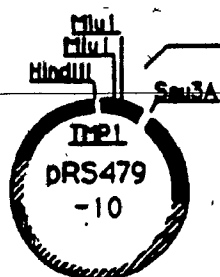
Once the 5' region of IMP1 was shown to confer periodic expression on IMP1-lacZ fusions in yeast, a preliminary mapping study of the IMP1 5' region was conducted to localize the information responsible for this regulation. There conveniently existed two MluI cut sites in the 5' regulatory region, which formed part of a repeated sequence (Figure 3). I constructed deletions in the region 5' to putative transcriptional and translational start sites by MluI restriction digestion of the IMP1 5' region in plasmids containing the entire IMP1 gene (Figure 9). Each of these plasmids, pRS479 and pRS593, was shown to complement cdc21-1 yeast as shown by growth of pRS479- or pRS593-transformed cdc21 strains at the restrictive temperature, suggesting that the plasmid IMP1 genes were functional.

MluI cuts only in the IMP1 5' region of these plasmids at the sites shown in Figure 3, and so the pRS479 and pRS593 digests were easily ligated as depicted in Figure 9. Derivative plasmids were isolated from transformed JF1754 and characterized by restriction endonuclease

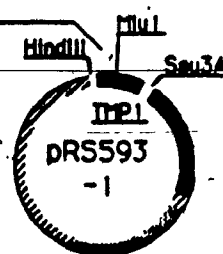
Figure 9: Strategy for the construction of normal and altered IMP1 5' regulatory regions, and for fusing these to lacZ. Starting with the plasmids pRS479 (Poon, unpublished) and pRS593 (Calmels, unpublished), alterations were induced in the 5' region of IMP1. These two plasmids, each of which complements a yeast cdc21 mutation at 37°C (unpublished), were used because their smaller size facilitated subcloning, and because no MluI sites were present other than at IMP1. In this example both plasmids have been shown cleaved with MluI (see Figure 1). Following religation of the digestion mix, derivatives subcloned in E. coli were screened for the presence of alterations (Figure 8), then cut with HindIII and Sau3A and inserted into pRS264 cut with HindIII and BamHI, as described in Figure 5.



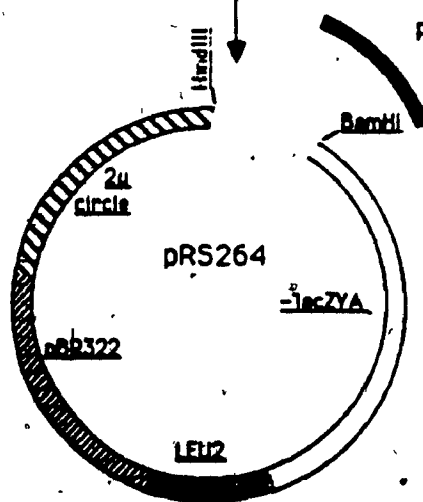
Cut with MluI and ligate



Mlu fragment inverted



Mlu fragment deleted



Replace Hind-Bam pRS264 fragment with Hind-Sau from altered pRS593' or pRS479'

digestion for plasmid integrity and loss or retention of the MluI fragment (Figure 10A). Of isolates which were further characterized, only pRS593-1 appeared to be missing only the 37 bp MluI fragment, whereas DNA sequencing showed that pRS479-10 contained an inverted "MluI" region (Taylor, pers. comm.).

C.2.2.b. Fusions of Rearranged Regulatory Regions to lacZ

The altered IMP1 HindIII -Sau3A regions from pRS479-10 and pRS593-1, respectively, were used to replace the normal IMP1 5' region in front of the pRS264 -lacZ HindIII -Sau3A double digests of these plasmids were mixed separately with HindIII -BamHI digested pRS264, followed by ligation and transformation (Figure 9). These pRS535 "derivatives" were initially characterized by restriction endonuclease mapping (Figure 10B). The relevant plasmid constructions are designated as pRS535-10C (derived from pRS479-10) versus pRS535-1A (from pRS593-1) and pRS535-7A (derived from pRS593-7). pRS535-7A appeared to be identical to pRS269, due the fortuitous retention of the extra Sau3A -BglII fragment (presumably derived from pRS264 DNA) also contained in pRS269. This conclusion was based both on restriction digests and on the pRS269-associated Bgal activity phenotypes; see Table 4). Most importantly, Figure 10B shows that the low MW MluI band missing from pRS593-1 (Figure 10A) is also missing in the derived pRS535-1A. DNA sequencing of these derivatives (G. Taylor, pers. comm.) confirmed that pRS535-1A was deleted for the information between the two MluI sites in the IMP1 5' region (Figure 11), and that this same information was inverted in pRS535-10C.

Figure 10. Detection of plasmids containing alterations at the 37 bp 5' IMP1 MluI fragment.

- A. Screening presumptive pRS535 and pRS479 derivatives. DNA from transformants derived from pRS479 or pRS593 as described in the text were pre-screened on 2% agarose slab gels with PvuII-PstI double digests. Digests which showed alterations of the PstI-PvuII fragment which had contained the MluI region were redigested with MluI. The MluI sites were end-labelled with α -³²P-dATP using the Klenow fragment of DNA polymerase (Maniatis, 1982, p.380). End-labelled fragments were loaded on 4% polyacrilamide gels. Two dye markers, bromophenol blue and xylene cyanol, which ran at 80 bp and 360 bp respectively at this percentage of gel, were used to estimate the MWs of small fragments. Shown is a photograph of the lower bands detected on the autoradiogram. Digests are of putative pRS479 derivatives (lanes 1 and 2); pRS479 (lane 3); pRS593 (lane 4); and putative 593 derivatives (lanes 5, 6 and 7). The DNA in lane 5 was not digested with MluI in order to detect the Pst-Pvu fragment (lane 5, faint band second from the top) from which was derived the predicted MluI fragments (lanes 3 and 4, lower 3 bands; see map in Figure 6) in IMP1 DNA. The lower band (arrow) contains the 37 bp MluI fragment, the band second from the bottom contains the 115 bp 5' Pst-Mlu fragment, while the third band up contains the 240 bp Mlu-Pvu fragment which includes the IMP1 translation start site. The 37 bp band (arrow) was not detected in lanes 2 and 6, but a longer exposure detected this band in lane 1. The isolates in lanes 1 and 6 were designated pRS593-1 and pRS479-10, respectively.
- B. Screening pRS535 derivatives for the absence of the 37 bp MluI fragment. DNA from transformants isolated as described in the text was pre-screened on 2% agarose slab gels with ClaI digests. Digests which showed alterations of the small IMP1-lacZ ClaI fragment containing the "Mlu" region were redigested with MluI. All cut sites were end-labelled and the digests were loaded onto an 8% polyacrilamide gel. The bromophenol blue and xylene cyanol dye markers ran at roughly 40 bp and 160 bp, respectively at this percentage of gel, and were used to estimate DNA sizes. The bottom band (arrow) contains the 37 bp MluI fragment, while the second band from the bottom contains the 115 bp Cla-Mlu fragment. Digests are of pRS535-10C from pRS479-10 (Lane 1); pRS264 (lane 2), pRS535 (lane 3), pRS269 (lane 4); pRS535-1A from pRS593-1 (lane 7 contained an unstudied derivative).

A

1 2 3 4 5 6 7

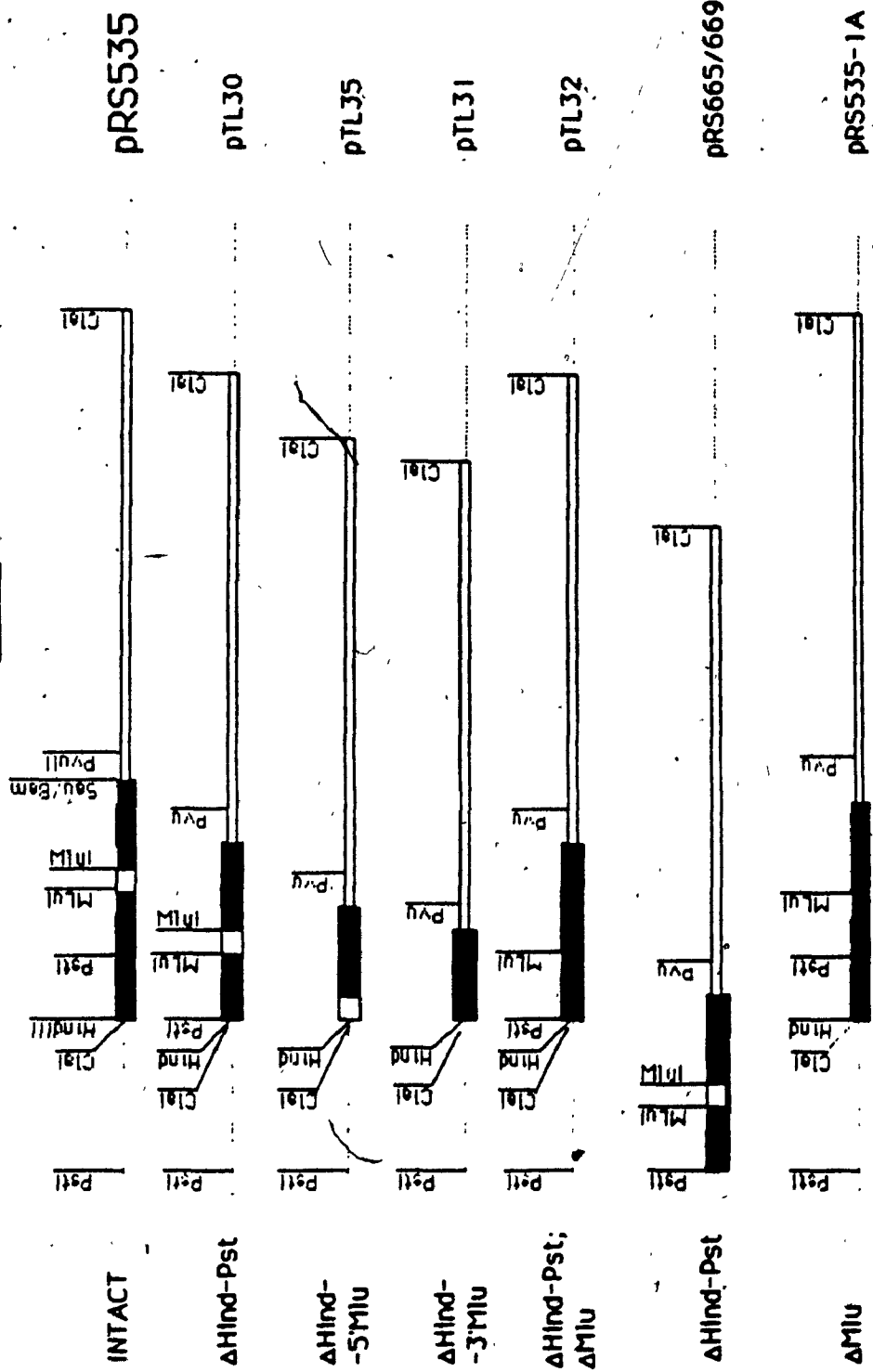
**B**

1 2 3 4 5 6

Figure 1.1. Linear maps of the IMP1-lacZ fusions used to study periodically regulated *Dgal* expression in yeast. The structure of these plasmids has been confirmed by DNA-sequencing (Taylor, unpublished). Narrow open rectangles denote lacZ sequences, thick black rectangles represent IMP1 sequences and the MluI region (see Figure 3), when present, is indicated by an open box. Sequences are aligned to the PstI site found in 2 μ -circle DNA, with the specific alteration given to the left of the map and the plasmid number to the right of the map ("Δ" indicates a deletion). The plasmid pRS535-10C map (not shown) is identical to pRS535, since this plasmid contains the MluI fragment in inverted orientation. One other plasmid isolated, but not shown, in this series, pRS535-7A is identical to pRS269 (Figure 5B), and the MluI fragment is still present in this construction.

-lacZ

TMP1:-



C.2.2.c. The pTL Series.

A representation of the various pRS535 derivatives used to map the IMP1 5' region is also given in Figure 11. The pTL30, 31,32,33 and 35 plasmids, provided by G. Taylor, were also created by altering the IMP1 5' region cloned into the pUC9 (Vieira and Messing, 1982) plasmid (G. Taylor, Ph.D. thesis), prior to fusing the HindIII - Sau3A fragment of this region to the pRS264 lacZ. The plasmids are therefore identical to pRS535 (Figure 6), except in the region 5' to the lacZ sequences (Figure 11). The known structure of the IMP1 5' deletions from the pTL series as well as the pRS "Mlu" mutations (all confirmed by G. Taylor, with the exception of pTL35) and observed function (see below) justify their being referred to as pRS535 "derivatives".

C.2.3. Transformation of pRS535 and its derivatives into yeast.

The plasmids described above have all been transformed into yeast strain AH22. Transformants were isolated by selecting for leucine prototrophs. In preliminary experiments, yeast strains LL20 and AH22 were transformed with pYT760-RYP3 (Figure 8), pRS264, pRS269 and pRS535 (Figure 5), as well as pRS58 (Figure 4A) which contained no yeast promoter elements. Several transformants were screened for β gal activity by ONPG assays. Transformants bearing pRS258, pRS269 and pRS535 gave similar high activities (> 100 pmol/min. per 10^7 cells in the ONPG assay) in both yeast strains, while pRS58 and pRS264 yielded no colour. These observations suggest that a yeast promoter (lacking in pRS58) is required to express lacZ in yeast, and that lacZ expression is facilitated by the IMP1 sequences. The inefficient expression of the pRS264 fusion gene is consistent with the DNA sequences of the IMP1

gene and of the lacZ gene which predicts that pRS264 would have a fusion gene with a (+1) frameshift at the junction between the IMP1 and the lacZ sequences. The junction sequences of pRS269 (the IMP1 information 5' to the BglII site fused to lacZ) and pRS535 (the IMP1 upstream Sau3A site fused to lacZ) were predicted and confirmed to be in-frame based on the above observations and by sequencing in the case of pRS535 (Taylor, unpubl., Calmels, unpubl.).

C.2.3.a. Frequencies and Stabilities of Transformants.

Table 3 gives the transformation frequencies of some of the constructed plasmids and their stabilities in yeast, as measured by the retention of the LEU2 marker when it is not selected for. High transformation frequencies (> 500 colonies/ μ g DNA), and loss of LEU2 during non-selective growth of the transformant suggest that the plasmid is replicating autonomously (due to the presence of the known ARS in 2 μ -circle DNA). Relatively low transformation frequencies and retention of LEU2 during non-selective growth indicate that the pRS665 plasmid deleted for the 2 μ -circle DNA has been forced to integrate into yeast chromosomal DNA in order to be maintained. Also shown in Table 3 are T.S. activities (P. Poon, pers. comm.) of the original transformants. Based on these activities and on the similar growth rates of these transformants (data not shown), the presence of the fusion plasmids was not deleterious to the strain studied. In addition, the plasmid sequences do not appear to be drastically altered in yeast, since restriction digests of recovered pRS535 DNA from yeast are indistinguishable from the original plasmids (L. Lee, unpubl.).

C.2.3.b. ARS sequences in plasmids used to transform AH22.

Based on the criteria outlined above, the data shown in Table 3 suggest that pRS258, pRS264, pRS269 and pRS535 replicate autonomously as

TABLE 3. Transformation frequencies, stability and lacZ expression in yeast strain AH22 of control plasmids used in this study.

Plasmid	Relative transformation frequency ^a	Stability ^b of transformants	Site of integration	Enzyme activity	
				T.S. ^c	β gal ^d
none	NA ^e	NA	NA	3.9	< 1
pRS264	1.0	0.48	NA	1.6	3.6
pRS269	0.8	0.53	NA	7.4	491
pRS535	0.9	0.20	NA	2.5	152
pRS665	0.001	0.98	<u>leu2</u>	ND ^f	17
pYT760-RYP3	1.1	0.60	NA	3.5	234

(a) Number of leucine prototrophs obtained in AH22 per μ g of plasmid DNA, relative to the number obtained with pRS264 (1520 colonies/ μ g).

(b) Proportion of leucine prototrophs remaining after 12 generations of growth without selection for the plasmid LEU2 marker, in YEPD.

(c) Thymidylate synthase (T.S.) activity expressed as pmol of ^3H released per minute per 10^8 log phase cells. Each value shown is an average of two determinations, and duplicate values were within 5% of one another (courtesy P. Poon).

(d) β -galactosidase (β gal) activity expressed as pmol of nitrophenol formed per minute per 10^7 log phase cells. Each value shown is an average of two determinations, and duplicate values were within 5% of one another.

(e) NA. Not Applicable.

(f) ND. Not determined.

circular plasmids in yeast. This was expected, based on the presence of yeast 2 μ -circle sequences on these plasmids.

On the other hand, pRS665 exhibits the characteristics of an integrating plasmid, including low transformation efficiency and stable transmission (of the plasmid-borne LEU2 marker). The low Bgal activity seen for pRS665 transformants further suggests that the plasmid exists at a low copy number, as expected for an integrated plasmid. Finally, Southern analysis of two pRS665 transformants indicated that both were integrated in high MW (chromosomal) DNA. One of these pRS665 plasmids, integrated at LEU2 (pRS665b-AH22; Lee, unpubl), was used for subsequent studies (see below).

C.3. Bgal Activities of pRS535 Transformants in Yeast.

C.3.1. Characterization of the pRS535 fusion enzyme in permeabilized AH22 cells.

Bgal activity appeared to accumulate more slowly than the rates predicted based on estimates of substrate turnover times for the native E. coli enzyme and on estimates of the amount of IMP1 TS gene product produced per cell (Bisson and Thorner, 1981; see Discussion). Changes in the efficiencies of transcription and translation of the foreign lacZ gene in yeast, or altered catalytic activity of the B-galactosidase moiety caused by the additional TS peptides, were not unexpected. However, it was essential for the experiments planned here that the stability of Bgal activity was constant under all conditions, in order to detect periodic changes in transcript levels as opposed to periodic degradation. Since initial observations suggested that Bgal activity in the pRS269-AH22 strain was unstable compared with the pRS258-AH22 control strain (compare Figure 23 with Figure 19), it was reasoned that the instability

Figure 12A. Bgal activity versus cell concentration from AH22 cells containing pRS535 grown in minimal media. Duplicate samples containing from 0.1 to 8 ml of cells growing logarithmically at a density of 1.06×10^7 cells/ml (OD600=0.267) were assayed using $N_2(1)$ /Brij permeabilization. Incubation time was 108 minutes, which yielded an average activity of 134 pmol/min per 10^7 , or 532 pmol/min per OD600.

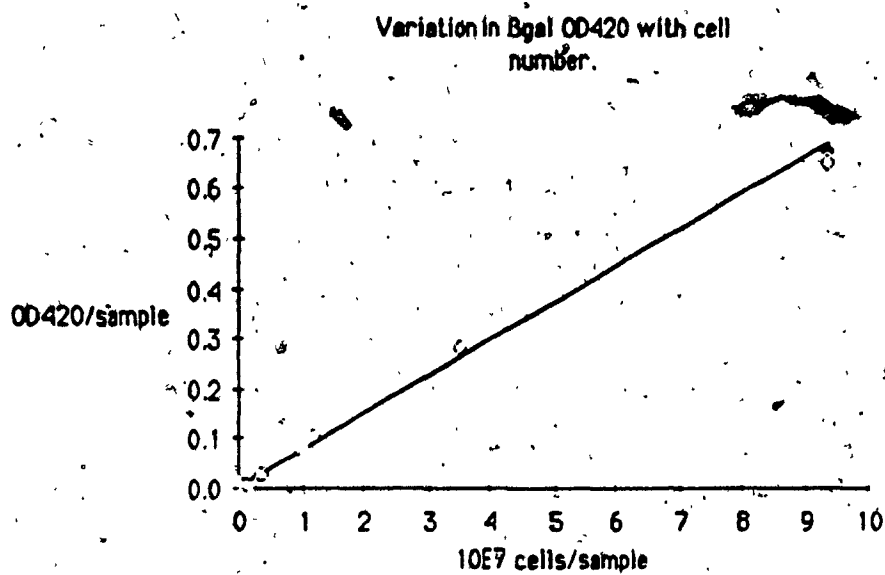
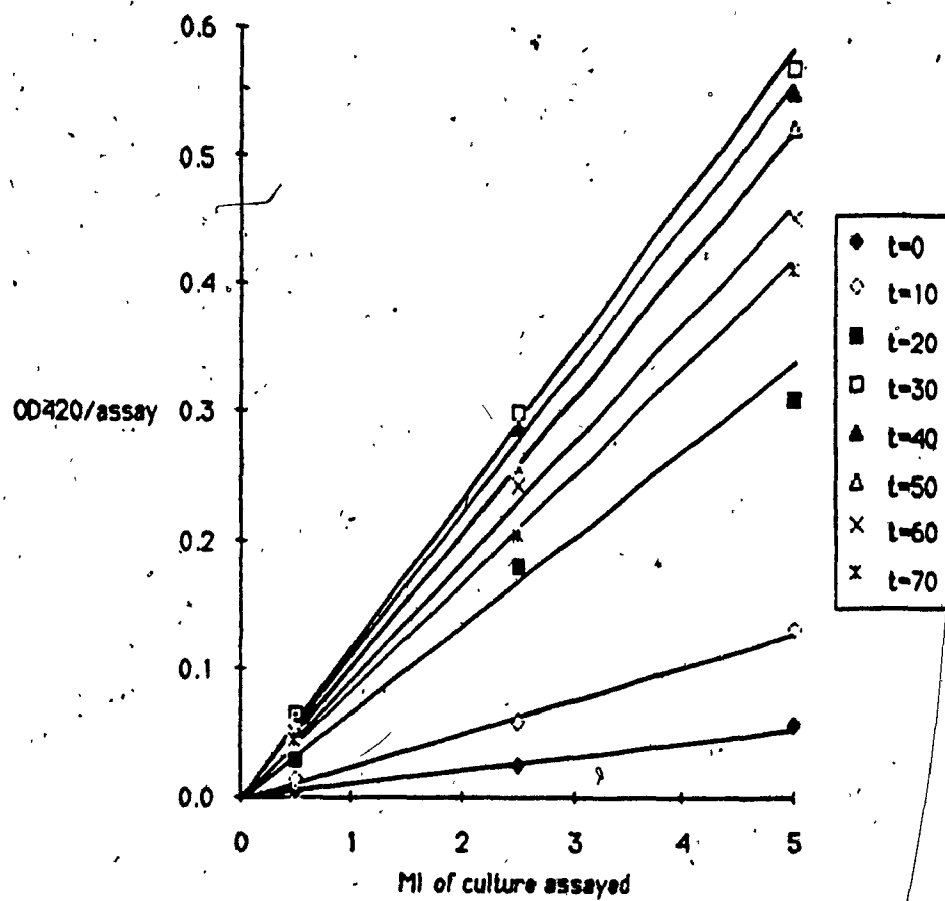


Figure 128. β gal activity versus cell concentration from synchronized AH22 cells containing pRS269. Samples containing from 0.5, 2.5 or 5 ml of cells taken at 10 minute intervals during synchronous growth were assayed for β gal activity using $N_2(1)$ /Brij permeabilization. Assay incubation time was 60 minutes. The 70 minute sample, at a density of 6.6×10^6 cells/ml at $t=70$ yielded an average activity of 515 pmol/min per 10^7 cells. It was noted during this experiment that omission of the Brij reduced detected activity by approximately 1/3.



might be inherent in the TS portion of the fusion protein. Therefore the expression in yeast of the pRS535 TS'-Bgal fusion protein, which contained only 13 N-terminal amino acids from TS, was compared with the pRS269 product for stability of expression. The results reported below indicate that the activity expressed from the pRS535 fusion gene product is consistently stable under the conditions used here, whereas the pRS269 fusion product's activity is unstable.

C.3.1.a. Bgal activity is directly proportional to cell number.

The Bgal activity expressed by pRS535-transformed log phase AH22 cells increases linearly with cell number from 10^6 to 10^8 cells per assay (Figure 12A). In addition, a preliminary kinetic study with pRS269-transformed AH22 indicated that Bgal activity from the latter plasmid increased in a linear fashion with cell density regardless of when in the cell cycle samples were taken (Figure 12B). Based on these observations, it was assumed that Bgal activity in these transformants is proportional to cell number during the cell cycle, over the range of activity levels tested.

C.3.1.b. Substrate (ONPG) converted in permeabilized pRS535-AH22 is directly proportional to the time of assay incubation.

The effect of assay time on the amount of substrate converted was performed on pRS269- and pRS535-transformed AH22 cells. The amount of substrate converted by pRS269-AH22 cells increased linearly for about 200 minutes (Figure 13A). Inclusion of the protease inhibitor PMSF did not alter the profile. In contrast, activity from pRS535-AH22 increased linearly for over 1000 minutes, in cells from minimal (Figure 13A) or rich medium (Figure 13B). This result meant that derivatives of pRS535 yielding very low Bgal activities in yeast could be directly

Figure 13A. Amount of ONPG substrate converted versus assay time, by permeabilized pRS535-AH22 (closed squares) or pRS269-AH22 (open squares; a separate experiment). Assays were of log phase cells, grown under selective conditions prior to harvesting. From pRS535-AH22, duplicate 0.3 ml samples of cells from a culture harvested at 1.0×10^7 cells/ml were assayed per time point. From pRS269-AH22, 10 ml samples of cells from a culture harvested at 1.1×10^6 cells/ml were assayed at each time point.

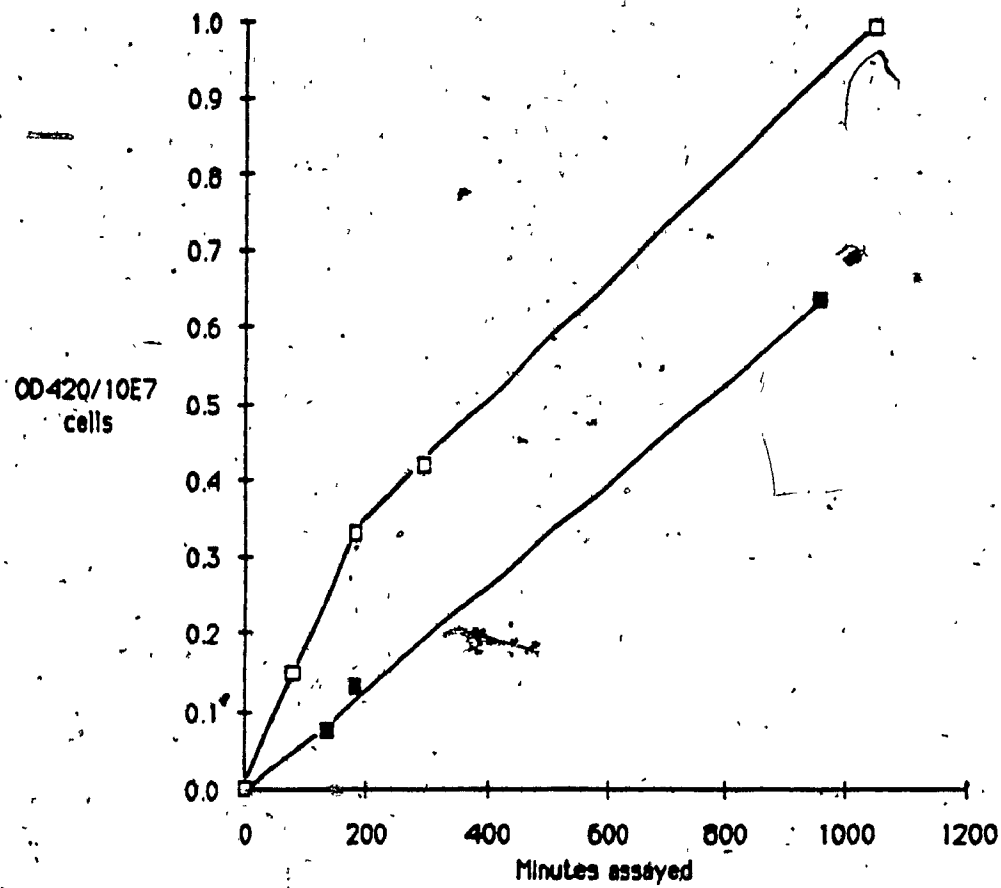
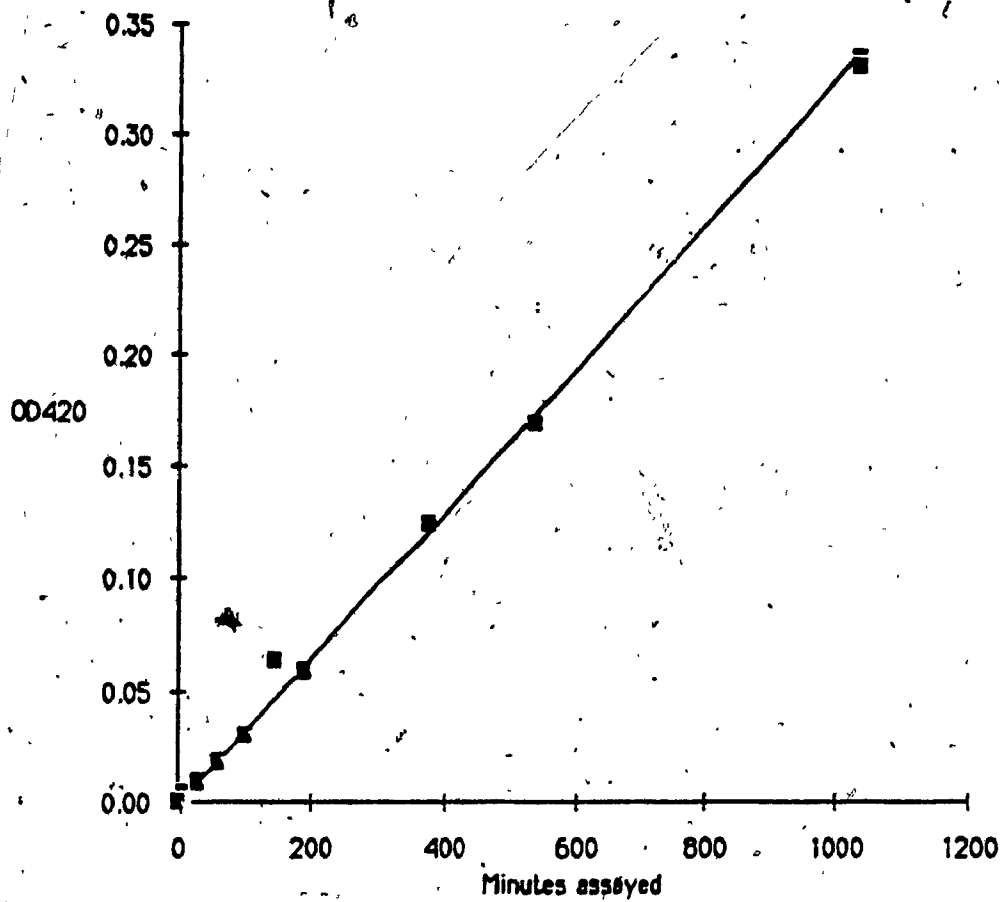


Figure 13B. β gal activity versus assay time from log phase AH22 cells containing pRS535 growing in synchrony medium. Duplicate 1.5 ml samples of cells from a culture harvested at 0.96×10^7 cells/ml were assayed per time point (N_2 /Brij permeabilization was used for the experiments shown in Figures 13A and B).

pRS535-AH22



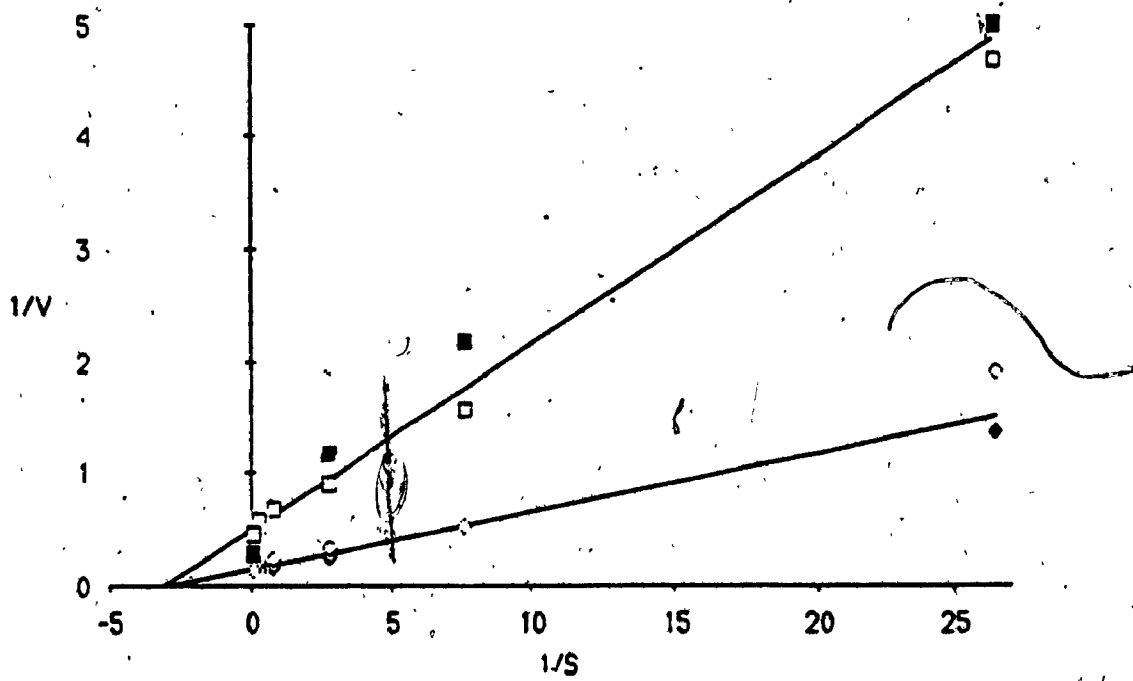
compared with the activity from the pRS535 "parent". However, it was noted that the curve did not extrapolate to zero in this experiment, possibly due to a low blank value. Following this observation, blanks had 50 μ l of medium (the approximate equivalent of one drop also found in the samples) added. Following this "correction", OD values of greater than 0.01 could be reliably reproduced between duplicate samples taken from a given experiment.

C.3.1.c. *Bgal* activities from the cell lysates show Michaelis-Menton kinetics.

Although the cell lysates used here formed only a crude system for "protein fusion enzymology", when enzyme activity was plotted against ONPG substrate concentration the values were linear on a Lineweaver-Burke plot (Figure 14). The experiment was repeated once, with similar results.

The K_m 's were identical for the AH22-pRS535 enzyme and the pRS269 enzyme (0.26 mM vs 0.25 mM), as might be expected for substrate binding to identical *B*-galactosidase active sites. This value approximated that found for the natural *E. coli* enzyme (0.18 mM; Kuby and Lardy, 1952). However, the V_{max} 's observed were different for the two fusion enzymes, averaging 5.3 nM/min.mg (cellular protein) from pRS269 versus an average 1.9 nM/min.mg from pRS535 (see Figure 14; mg protein was estimated from cell counts). The V_{max} 's at two different cell concentrations were very similar within strains. V_{max} from both strains was considerably lower than for the purified *E. coli* enzyme (32 μ M/min). These differences, due to different amounts of active enzyme, can be used to estimate quantities of enzyme (assuming full activity; Schleif and Wensink, 1981). For example, the above results suggest that active,

Figure 14. Lineweaver-Burke plot of BgaT activity as $1/v$ (v in pmol/min.mg. protein) versus $1/[S]$ (where $[S]$ is ONPG substrate concentration in mM) from log phase AH22 cells containing pRS535 (diamonds) or pRS269 (squares), and growing in selective medium. Open symbols designate samples isolated at higher culture densities, while closed symbols designate samples isolated from cultures at lower cell densities. Protein values were estimated from duplicate cell counts, based on an estimate of 0.578 mg of total cellular protein per 10^8 cells. High culture density was 1.6×10^7 /ml, while the low culture density was 0.30×10^7 /ml. OD420 values were based on a blank made up at 4mg/ml ONPG. Note that the ONPG concentration of 4 mg/ml (13.3 mM) used in this study equalled 0.075 mM^{-1} as $1/[S]$.



plasmid encoded β -gal enzymes comprise roughly 0.01% of total cellular protein, and that pRS269-AH22 produces (3X) more active β -gal than pRA535-AH22.

At very high [S]s, the reaction rate from pRS535 appeared to be slightly higher than the extrapolated V_{max} (data not shown), suggesting that exposure of cellular β -galactosidase to substrate becomes a limiting factor at lower substrate concentrations. For this reason, the ONPG concentrations were kept at high levels (≈ 3.7 mg/ml or 13.3 mM in the reaction mix) to optimize reaction velocities. This approach appeared to be valid, since the $V_{max}/10^7$ cell or /OD600 calculated from these data approximate the activities calculated in several experiments (Table 2, Table 4). The results imply that these lysates can probably be used for kinetic studies, although no subsequent studies were performed.

C.3.1.d. Other parameters contributing to activity. The pH optima of the β gal activities from the pRS535 fusion protein were compared with those from pRS269. As seen from Figure 15A, the pRS535-type activity was stable from pH6.5 to pH7.6. This range is much greater than the maximum pH variabilities in the assay buffer (≈ 0.2 pH) observed due to pH differences in the media. In practical terms, this means that the cells need not be washed prior to assay or freezing, which in turn allows for the rapid sampling which is required for the synchrony experiments discussed below. In comparison, the β gal activity from pRS269 falls off more sharply at higher or lower pH. However, this range is also sufficient to allow reproducible assays of unwashed samples of pRS269-AH22. By comparison, the native *E. coli* enzyme has a pH optimum of 7.2, and is stable from pH 6 to pH 8 (Kuby and Lady, 1952).

Figure 15A. *Bgal* activity (measured here in nmol/min.ml) as a function of pH in pRS535-AH22 (closed diamonds) and in pRS269-AH22 (open diamonds), in samples from late log phase cells. 5 ml of cells, isolated at a culture OD600 of ≈ 1 , were assayed per point. Note that the normal pH in a *Bgal* assay was 7-7.1, for this study.

Bgal activity vs pH

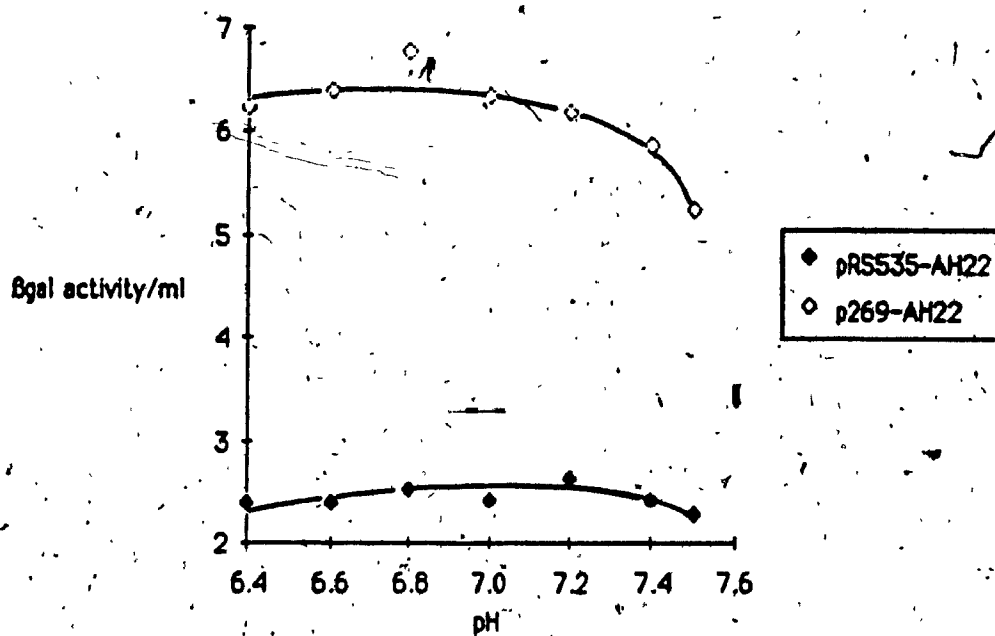
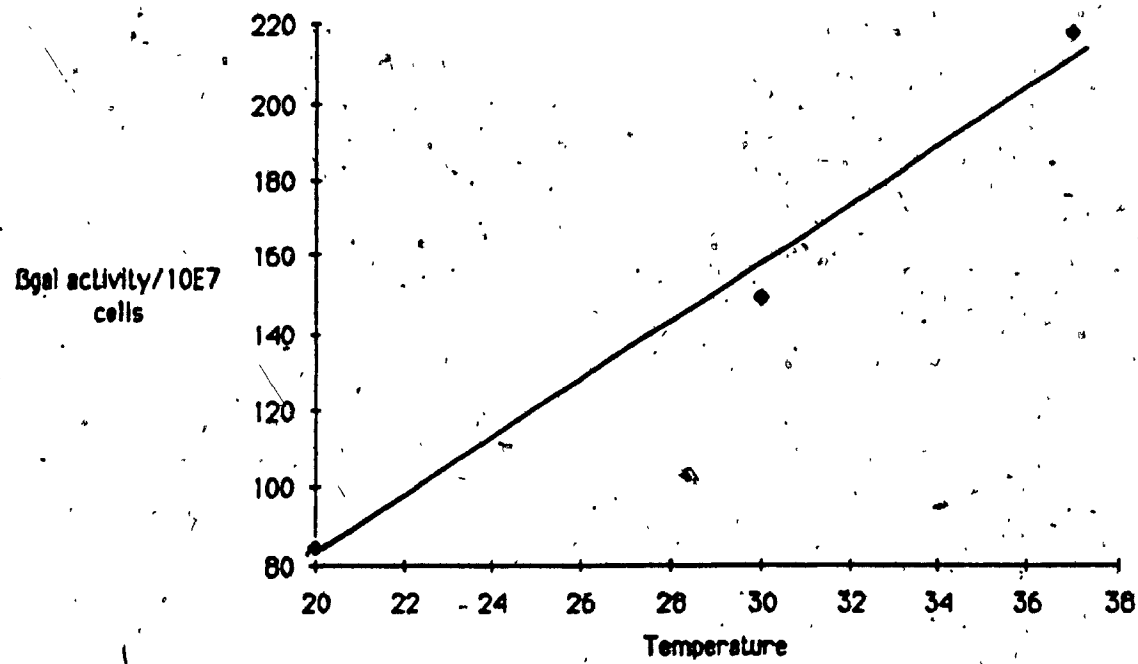


Figure 15B. Bgal activity as a function of temperature in PRS535-AH22 (samples from log phase cells). Duplicate 3 ml samples of cells, isolated at a culture density of $0.96 \times 10^7/\text{ml}$, were assayed per point using $\text{N}_2(1)/\text{Brij}$ permeabilization.

Bgal Activity/10E7 cells vs T°C

In practical terms, the variables of temperature and of initiating the assay contributed considerable uncertainty. When Bgal activity was assayed at three temperatures (Figure 15b), activity adhered fairly closely to Q10 values, doubling every 12°C. Such changes in the temperature of assay thus appeared to affect rates of catalysis and not enzyme stabilities.

For initiating the assay, preliminary experiments showed that one vigorous 20 second vortexing of a sample prior to incubation detected at least 95% of the activity detectable with prolonged vortexing. Brij/N₂(1) was about 5% less efficient at permeabilizing high plasmid copy number cells than was Chlf/SDS, but was 5% more efficient for cells with low plasmid copy number. Permeabilization was not improved by adding glass beads, or by repeated freeze-thaws (data not shown).

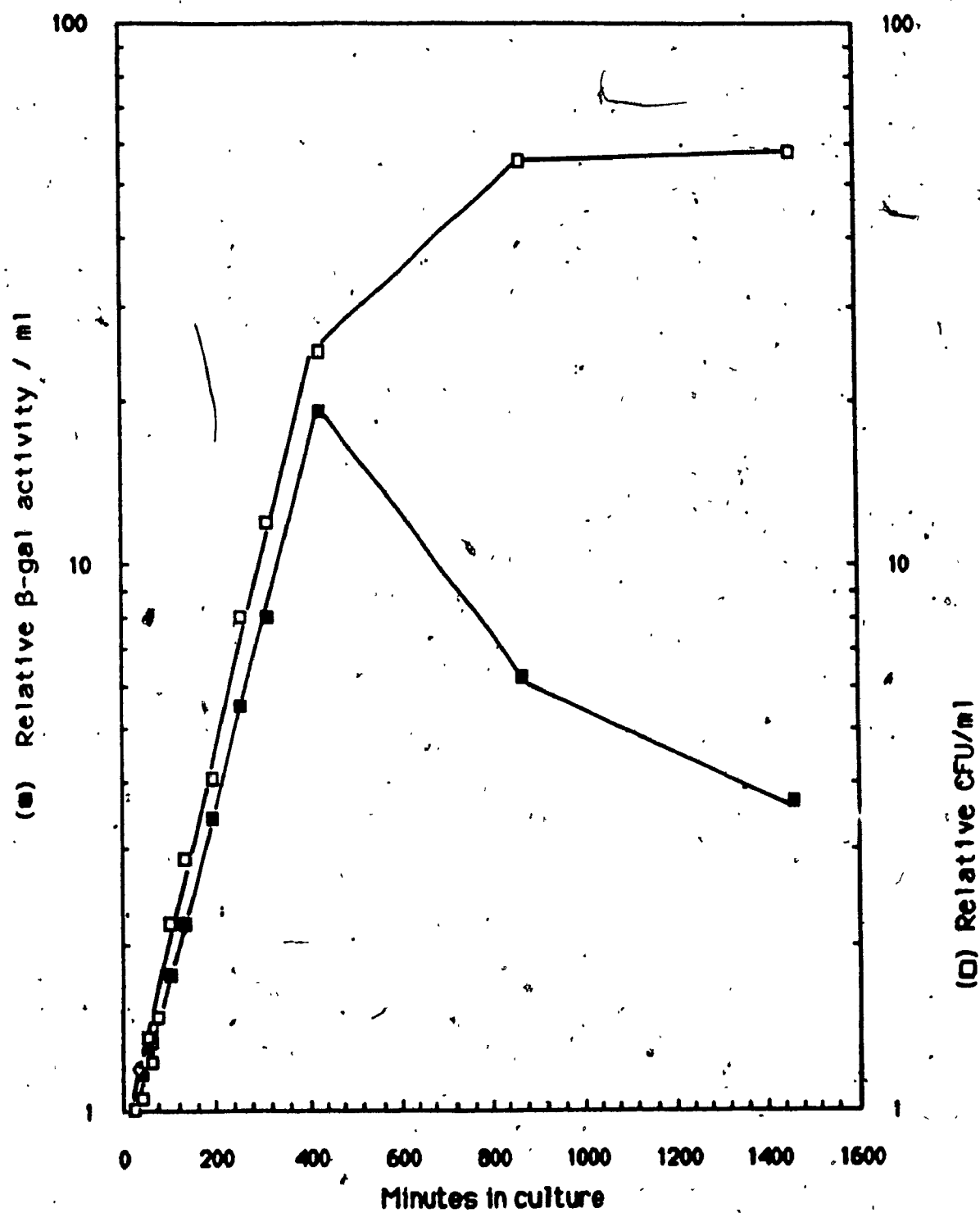
These data indicated that the pRS535 enzyme activity was sufficiently stable to use for examining the regulation of pRS535-AH22-derived IMP1'-lacZ fusions. It further supported the proposal of Greenwood (1986) ~~that~~ the thymidylate synthase moiety conferred instability on the pRS269 fusion protein's Bgal activity, and therefore in all likelihood, on the activity of thymidylate synthase itself.

C.3.2. Accumulation of Bgal Activity in Batch Cultures.

The Bgal activities observed from pRS535 are consistently lower than those from pRS269 and pYT760-RYP3 (Table 1, see also Table 4). While a difference might be expected for the latter plasmid due to the different promoter, it was unclear how the activity from pRS269 could be both unstable (Greenwood) yet higher than that from pRS535, since both activities should derive from the same promoter.

This phenomenon was also noted in initial studies of Bgal activities

Figure 16. β gal activity/ml of culture relative to $t=0$, compared with relative culture density (CFUs/ml), from pRS269-AH22 in 0.1%/2xYNBH medium (2% glucose). Note that log phase β gal activity increases only slightly more slowly than culture density in this medium, which is non-selective for the plasmid.



from pRS269-AH22 and pRS535-AH22 in YNBD batch cultures: In the first experiment, log phase pRS269-AH22 was resuspended in fresh medium (Figure 16), whereupon cell number and β gal activity both continued to increase exponentially. This result indicates that the pRS269 protein fusion is not sensitive to subculturing per se, during log phase. The five-fold decline in β gal activity per cell at the end of log phase was also observed by Greenwood (M.Sc. thesis).

However, when pRS269-AH22 cells pre-grown to stationary phase were subcultured into fresh medium, β gal activities increased up to 20-fold within 60 minutes (Figure 17a). The large increase in activity per culture OD600 was maintained until stationary phase, when activities fell at least 10-fold. The initial rapid rise in β gal activity early in the culture is likely due to a combination of the pRS269 fusion product instability in the stationary phase cells, and an induction of new activity. For example, Calmels (in preparation) has observed that IMP1 RNA increases dramatically and almost immediately when AH22 cells are stimulated to proliferate by inoculation into fresh medium.

In contrast, no dramatic increase was observed when stationary phase pRS535-AH22 cells were inoculated into fresh medium (Figure 17b). An increase of less than two-fold was observed over the same interval that β gal activity from pRS269-AH22 showed a 20-fold increase. In addition, there was almost no loss of activity in stationary phase in pRS535-AH22. The difference in activity between the two strains can be explained by the stable activity which is present in stationary phase pRS535-AH22 cells, and therefore is also present at the beginning of the culture as a high background activity which would hide a possible induction. However, the possibility that pRS269-AH22 unstable β gal activities are also more substantially induced when G_0 cells are

Figure 17A. β gal activity from pRS269 as a function of time in culture, when stationary phase AH22 cells containing pRS269 are inoculated into selective medium. The undiluted cell densities, in Klett units, are averaged from 8 cultures whose separate ODs were less than 5% from the mean value. Cells were previously grown into stationary phase (cell density of $1-2 \times 10^8$ /ml) in selective medium, then diluted 1/50, or 1/100 (experiment #2), into fresh selective medium. Cells in experiment #2 were grown in 2 x the normal glucose concentration (ie. 4%), in order to compare β gal metabolism through the growth cycle with the results of Greenwood (M.Sc. thesis). Time zero points are subject to some uncertainty, since the samples were isolated from the previous culture.

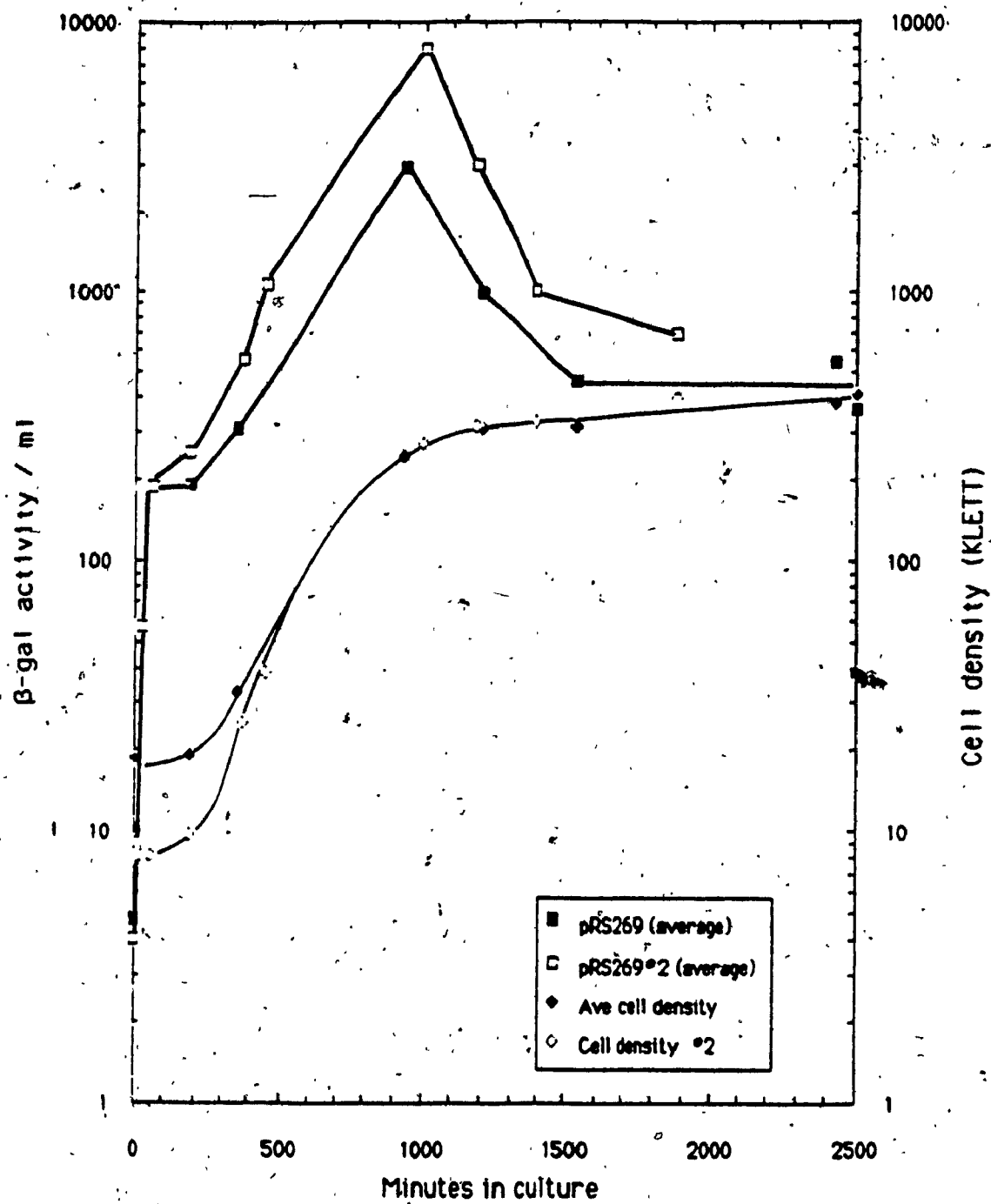
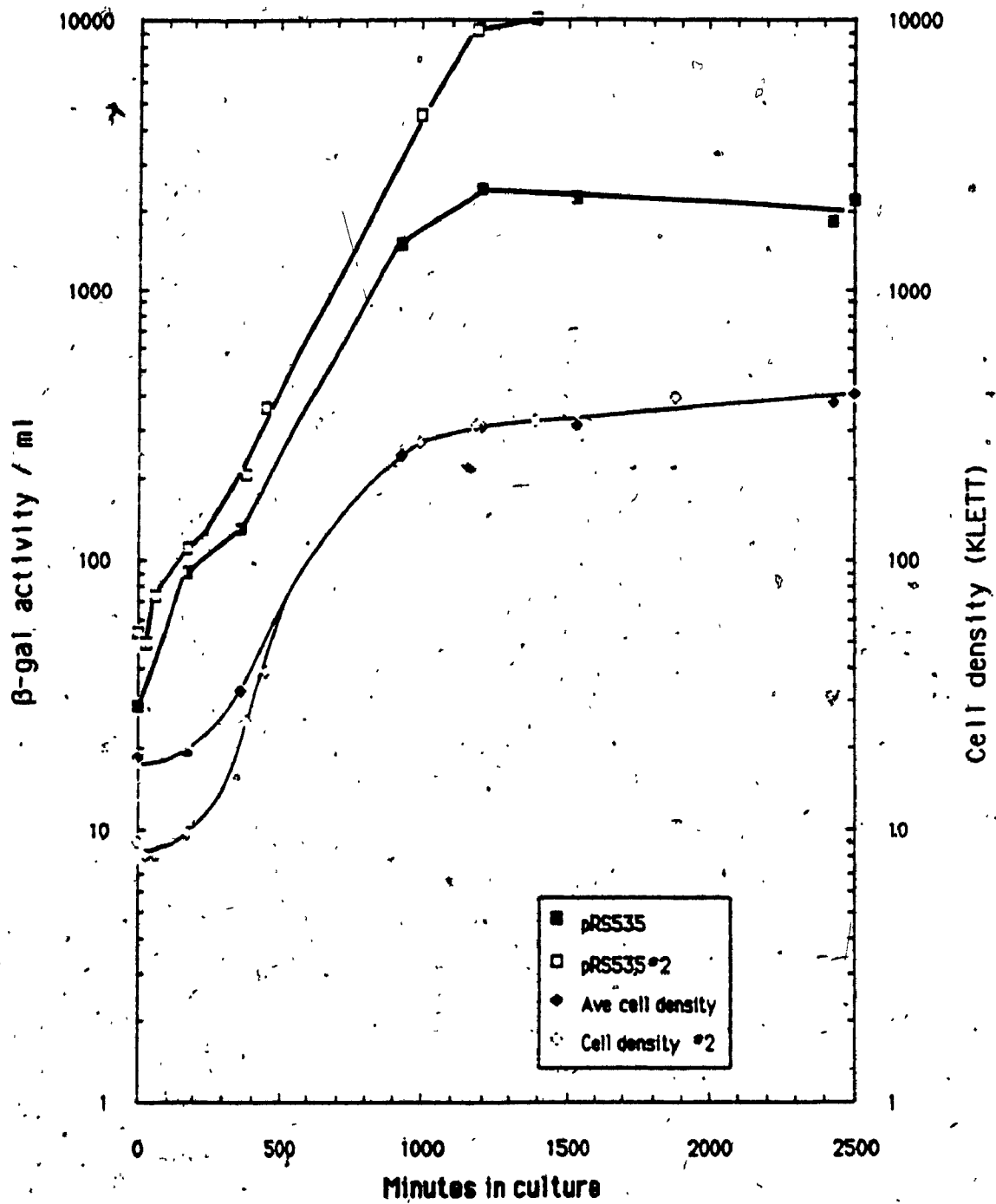


Figure 17B. β gal activity (squares) from pRS535 as a function of time in culture, when stationary phase AH22 cells containing pRS535 are inoculated into selective medium. The undiluted cell densities, in Klett units, are averaged from 8 cultures whose separate ODs varied by less than 5% from the mean value at a given point. Cells were previously grown into stationary phase (cell density of $1-2 \times 10^8$ /ml) in selective medium, then diluted 1/50 (open symbols) or 1/100 (closed symbols; experiment #2) into fresh selective medium. Cells in experiment #2 were grown in 2 x the normal glucose concentration (ie. 4%), in order to compare β gal metabolism through the growth cycle with those of Greenwood (M.Sc. thesis). Time zero points are subject to some uncertainty, since the samples were isolated from the previous culture.



induced to proliferate cannot be excluded. Most importantly, however, the pRS535 fusion apparently produces a Bgal activity of consistent stability at all stages in growth.

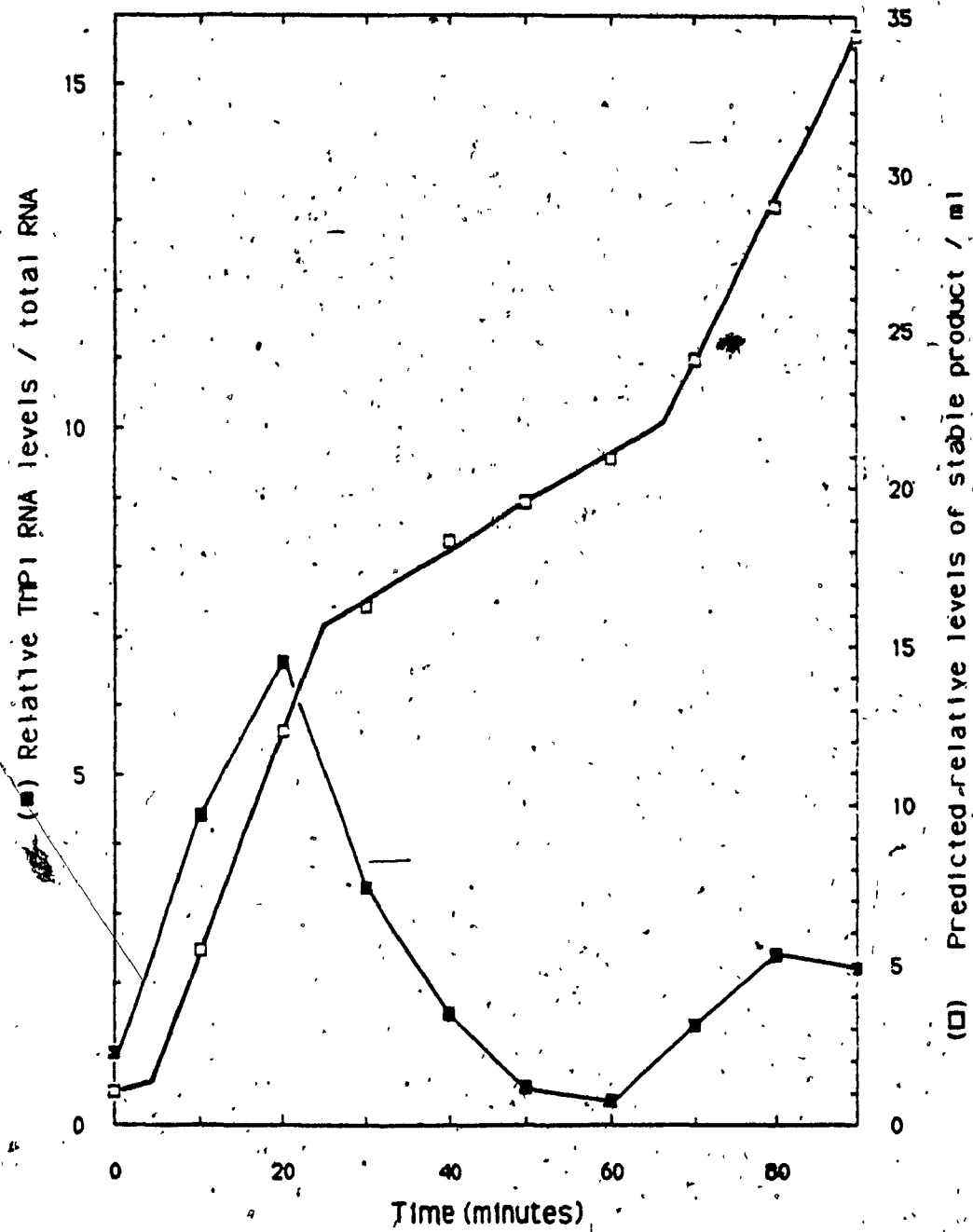
C.3.3: B-galactosidase Accumulation in Synchronized Cells.

Expression of the IMP1-lacZ fusion gene contained on pRS535 and shown to code for a stable Bgal activity, was then followed during synchronous growth of yeast strain pRS535-AH22. As noted by Storms et al (1985) and as depicted in Figure 18, IMP1 RNA levels detected by Northern analysis rise and fall over two synchronous rounds of replication, with a peak near the beginning of each S phase (replotted from Figure 2). Although the typically larger first generation peak could be due to induction of cell proliferation following α -factor arrest, it is assumed that the presence of the second RNA peak indicates true periodic expression. As a result, two generations of synchronized growth were monitored in some of the experiments described below, to detect true periodic Bgal activity fluctuations as opposed to simple induction curves.

The synchronous IMP1 RNA profile shown in Figure 18 was based on equal amounts of RNA loaded per sample. However, in the present study, cumulative Bgal activity profiles were monitored per ml of culture, following the procedure of Mitcheson (1971). This was simpler and more accurate than monitoring Bgal activity/mg total RNA or per cell. The increased error introduced with the second parameter is avoided, and in this instance, data per ml of culture is more informative for relative activity levels and for degree of synchrony.

If IMP1 RNA is unstable and doesn't accumulate, while the pRS535-directed Bgal activity is stable, the Bgal profile from pRS535 would be expected to approximate a step, rather than a peak (Mitcheson,

Figure 18. Relative levels of IMP1 RNA accumulated from synchronized AH22 cells during one cell cycle (dark squares; Storms et al, 1985). Equal concentrations of total cellular RNA were loaded onto an agarose gel, electrophoresed, then Northern-blotted and probed for IMP1 RNA (following the procedure of Thomas, 1983). The ~~same~~ relative levels are also plotted as accumulated values (open squares). In this transformation the data from a given time point is summed with the data from all previous time points. To partially correct for the expected two-fold, exponential increase in total RNA through the cell cycle, each point was then multiplied by the culture OD600 (taken from a separate synchronous culture of similar cell density) for that time. This manipulation yielded the predicted relative levels of stable product per ml of culture. In this culture G1 occupied the first 20 minutes, S phase occurred from 20-50 minutes, and G2, from 50-70 minutes. Cytokinesis and the following G1 occurred from 70-90 minutes.



1971). The shape of this step can be predicted from the observed RNA peaks shown in Figure 18, as follows. If most of the IMP1 RNA detected at a given sample time during the yeast cell cycle by Storms *et al* (1985, see Figure 18) is assumed to be degraded prior to the next sample, then the relative RNA levels appearing at each point should be due to new synthesis. If so, an estimate of the total amount of IMP1 RNA synthesized by time "t" in the cell cycle can then be made, by summing all of the RNA present up to and including time "t". By then plotting the cumulative levels of RNA obtained by each time point through the cell cycle, a step profile resembling that shown in Figure 18 is generated.

Assuming that expression from pRS535 is periodic, this "stable product" profile should resemble that of the stable β gal activity produced from pRS535 (see Figure 21), particularly if macromolecular (eg. RNA) synthesis is corrected for. For example, in Figure 18, the cumulative IMP1 RNA values were multiplied by the increasing culture OD600, at each time point, to roughly correct for relative increases in total RNA.

In other words, in synchronized cells the predicted periodic accumulation of β gal activity from pRS535 should resemble the cumulative IMP1 RNA synthesized, and both profiles should resemble the step profile seen (Storms *et al*; 1984) for DNA synthesis.

In these synchrony studies, the ratio of unbudded cells per total CFU (UB/CFU) was used to monitor the stage in the cell cycle of a particular sample (CFU or "colony forming units", counted via hemocytometer, consist of single cells or cells with buds which would give rise to one colony in an appropriately diluted plating test). In AH22, as in most yeast strains, UB/CFU decreases as buds form at the beginning of DNA synthesis (Storms *et al*; 1985) and increases again as cells undergo

cytokinesis. The correlation of bud emergence with onset of DNA synthesis was not affected by the presence of pRS535 (data not shown).

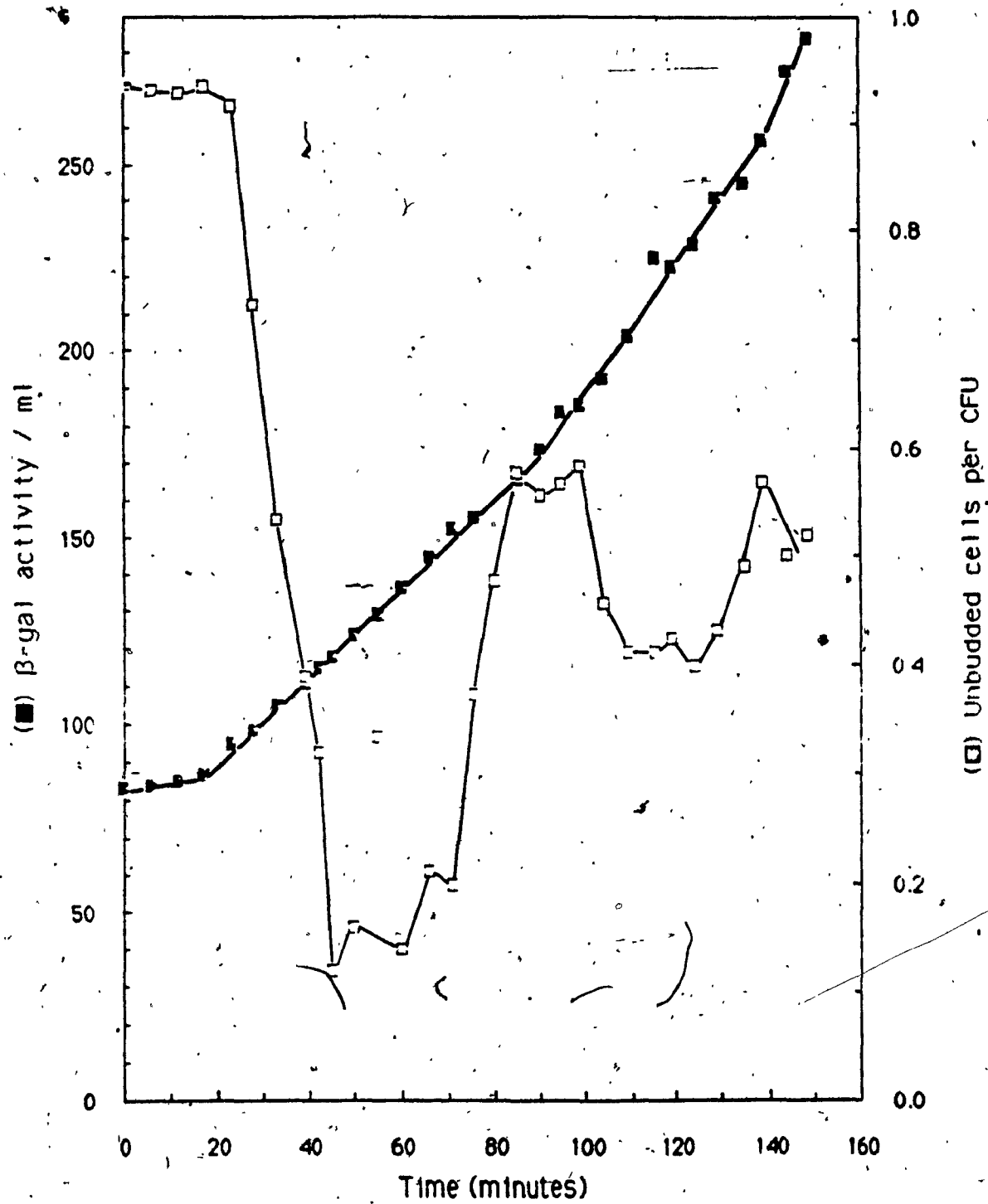
C.3.3.a. A non-periodic Bgal activity profile from pYT760-RYP3.

The Bgal activity profile during the cell cycle of synchronized pYT760-RYP3-AH22 cells is given in Figure 19. This RYP3-promoted activity showed a non-periodic, "continuous linear" profile very similar to that predicted and observed in synchronized *S. pombe* by Mitcheson (1971, p159-169) for the non-periodically expressed enzymes acid phosphatase, sucrase and alkaline phosphatase. As a result this strain has been retained as the non-cell-cycle regulated control for subsequent experiments.

It was noteworthy that preliminary attempts to prove that acid phosphatase activity also increased in a continuous linear manner in synchronized *S. cerevisiae* were unsuccessful, due mainly to sharp fluctuations in activity at both the beginning and end of the cycle.

For a definition of "not periodically expressed", it can be seen from Figure 19 that activity from pYT760-RYP3 increases linearly for one generation (one round of budding), then the slope of the increase changes. The activity continues to increase at the new rate until the end of the second generation, whereupon the slope increases again. This observation is consistent with an expected increase in the rate of synthesis due to the presence of newly replicated genes. The activity slope does not exactly double each generation, probably because the average degree of synchrony achievable in this system is 75% in the first generation and 60% in the second, based on the UB/CFU ratio.

Figure 19. "Continuous linear" Bgal activity profile from pYT760-RYP3, which contains an unknown yeast promoter fused to lacZ, in AH22, during 2 cell cycles of synchronous growth. The cell cycle is depicted by changes in the proportions of unbudded cells per CFU (open squares). In G1, proportions of unbudded cells are relatively high and constant, while during S this proportion decreases. During G2 the proportion of unbudded cells is relatively low and constant, then the proportion increases again at cytokinesis. Duplicate samples were assayed for Bgal activity from each time point using chloroform/SDS permeabilization. Duplicate sample OD420s did not deviate from the mean OD420 by more than 5%. Since the mean OD420 was used to calculate Bgal activity, this percent error can be directly applied to the activities shown here. A similar format is followed for subsequent Figures which show Bgal activities.

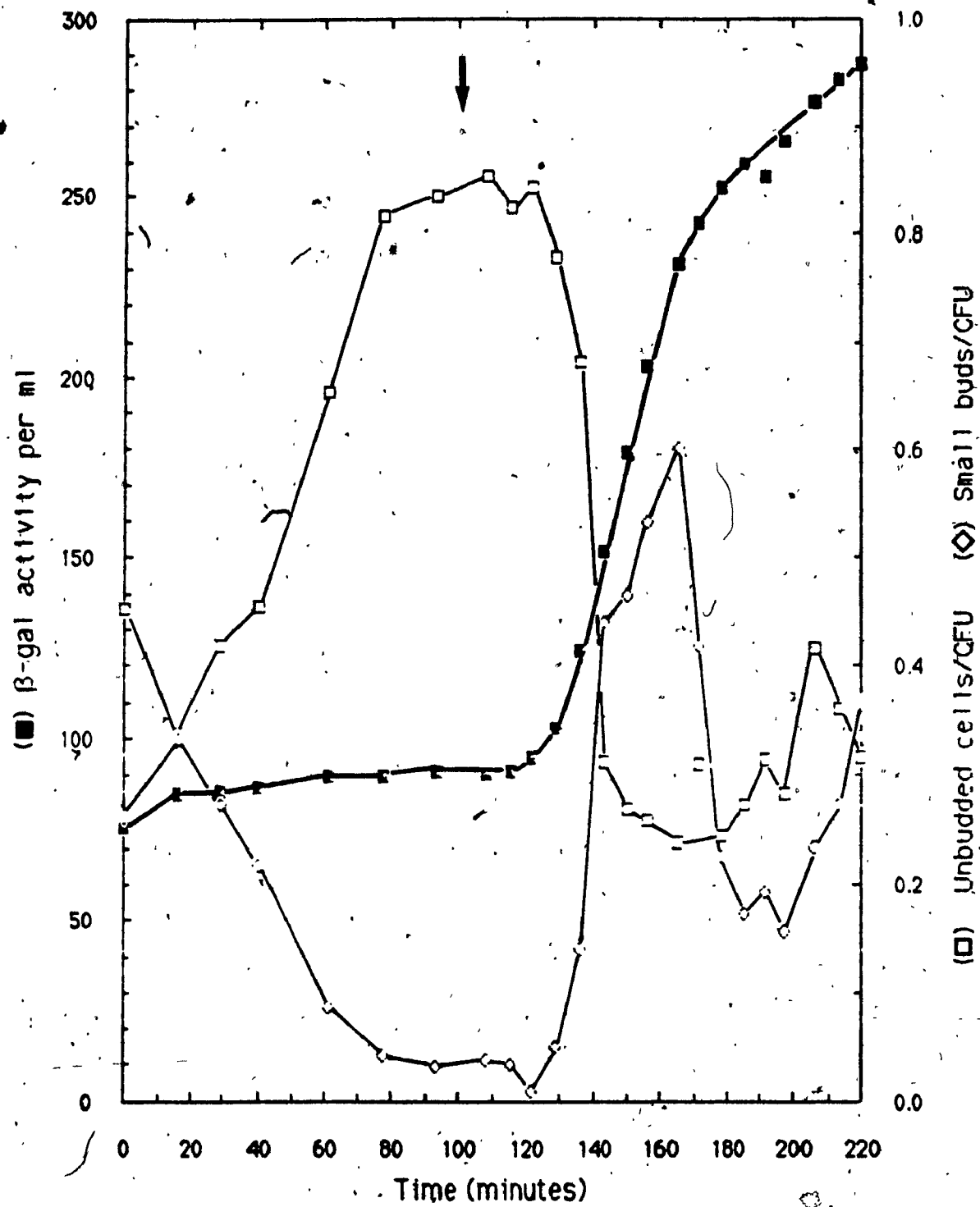


C.3.3.b. Periodic vs START-dependent activity profiles. In contrast to the obviously non-periodic *Bgal* activity profile from pYT760-RYP3, it is possible that the expression of some genes may be proliferation dependent, although not cell cycle-dependent. Such genes may be simply "turned up" due to the return of cells to a proliferative state (START-dependent regulation mediated by class I or class II start genes described in the Introduction) from a less active state during α -factor arrest or G_0 arrest, and then left on at some constant rate.

We can also determine whether a *lacZ* fusion gene whose mRNA accumulation has been shown to be non-periodic, such as the CYC1'-*lacZ* fusion gene (Osley *et al.*, 1986), is none-the-less START-regulated at the level of the transcript, by measuring the accumulation of *Bgal* activity from this fusion in synchronized cells. For example the expression of CYC1, the gene for cytochrome-c-oxidase, is START regulated to some extent. The linear *Bgal* accumulation predicted from the CYC1'-*lacZ* fusion RNA levels (*ibid*) is not observed. Instead, *Bgal* accumulation from the CYC1'-promoted -*lacZ* fusion of plasmid pL0669-2 transformed into yeast strain DBY747 accumulated in a START-dependent fashion during an α -factor arrest-and-synchrony experiment (Figure 20; no attempt was made to induce the fusion gene during this synchrony).

Note that *Bgal* activity from the CYC1 promoter does not accumulate in the presence of α -factor. The latter result was later repeated in a pRS535-like construction (Appendix). From Figure 20, it can be seen that following α -factor release, a three-fold induction of activity taking up most of the first budding cycle levels off to a lesser, apparently constitutive increase in the second generation. This potentially important observation suggests that START-dependent fluctuations in the expression of a gene product can be regulated at the level of the

Figure 20. A presumptive "induced-non periodic" Bgal activity profile from pLG669-2, which contains the CYC1 5' regulatory region fused to lacZ, in yeast strain DBY747, during 1 1/2 cell cycles of synchronous growth. In this experiment, Bgal activity was also monitored while the cells were arresting in α -factor (from $t=0$ to $t=100$), as well as after release from the α -factor block at $t=100$ (arrow). Here, the cell cycle is depicted as changes in the proportions of newly budded cells per CFU (open diamonds), since cytokinesis was delayed in this strain (detected as the prolonged G2 seen from the unbudded cells; open boxes). The cell cycle as monitored by new buds/CFU proceeds so that in G1, new bud proportions are low and constant, during S the proportion increases, and during G2 proportions decrease again. Duplicate 5 ml samples were assayed for Bgal activity from each time point using N_2 /Brij permeabilization. Sample OD420s did not deviate from the means shown by more than 5%. The synchrony medium was further supplemented with tryptophan, leucine and histidine, for which DBY747 is auxotrophic (the pLG plasmid relieves a fourth auxotrophy for uracil).



transcript (the CYC1'-'lacZ fusion gene product is stable), in the absence of START-dependent fluctuations in mRNA levels.

C.3.3.c. A periodic Bgl activity profile from synchronized pRS535-AH22 cells.

With the aforementioned caveats in mind, the most likely interpretation of the Bgl activity profile from synchronized pRS535-AH22 cells over two generations (Figure 21) is that the IMP1'-'lacZ fusion contained on this plasmid is periodically expressed. Unlike the profiles shown for Figures 19 and 20, this data mimics the step profile predicted from the IMP1 RNA profile shown in Figure 18. A nearly identical result was obtained when Brij 35/-80°C permeabilization was used (Figure 21, lower profile) instead of chloroform/SDS permeabilization. The two Bgl activity curves shown come from totally separate experiments, and so give some idea of the repeatability of the observation.

C.3.3.d. The pRS535 periodic Bgl profile is independent of genomic location.

There was some concern that the periodic expression observed might be an artifact of the plasmid-borne fusion gene, and so a yeast strain containing the pRS535 derivative pRS665 described above, integrated into the yeast chromosome at LEU2 (L. Lee, pers. comm.), was tested for its ability to regulate Bgl activity during synchronous growth. As shown in Figure 22, the activity profile from pRS665 closely resembled that from pRS535. The periodic profile produced from the IMP1 5' regulatory region is therefore independent of the 2 μ -circle ARS, and of the chromosomal location of the fusion gene. The Bgl activity of the

Figure 21. "Step" Bgal activity profile from pRS535-AH22 containing the intact IMP1 5' regulatory region fused to 'lacZ, during two cell cycles of synchronous growth. The Bgal data from two separate synchronies are shown. Cell cycles are depicted as changes in the proportions of unbudded cells per CFU, where 0:1 cells are maximally unbudded. Duplicate 2.5 ml samples were assayed for Bgal activity from each time point using either chloroform/SDS permeabilization (dark squares) or N₂(1)/Brij 35 permeabilization (dark triangles). Sample OD420s did not deviate from the means shown by more than 5%, except the 118 minute chloroform/SDS sample (8%) and the 6 minute N₂(1)/Brij sample (10%).

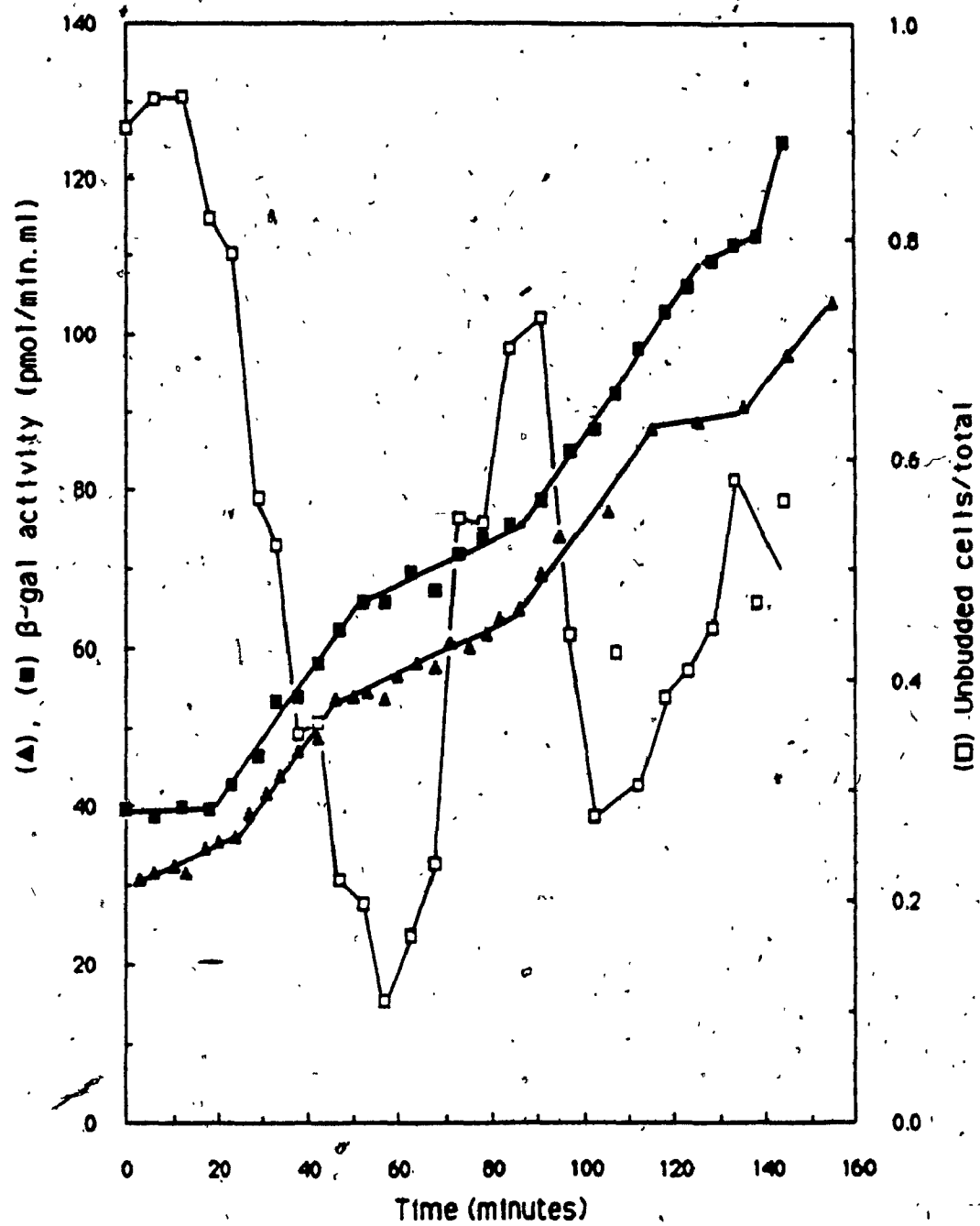
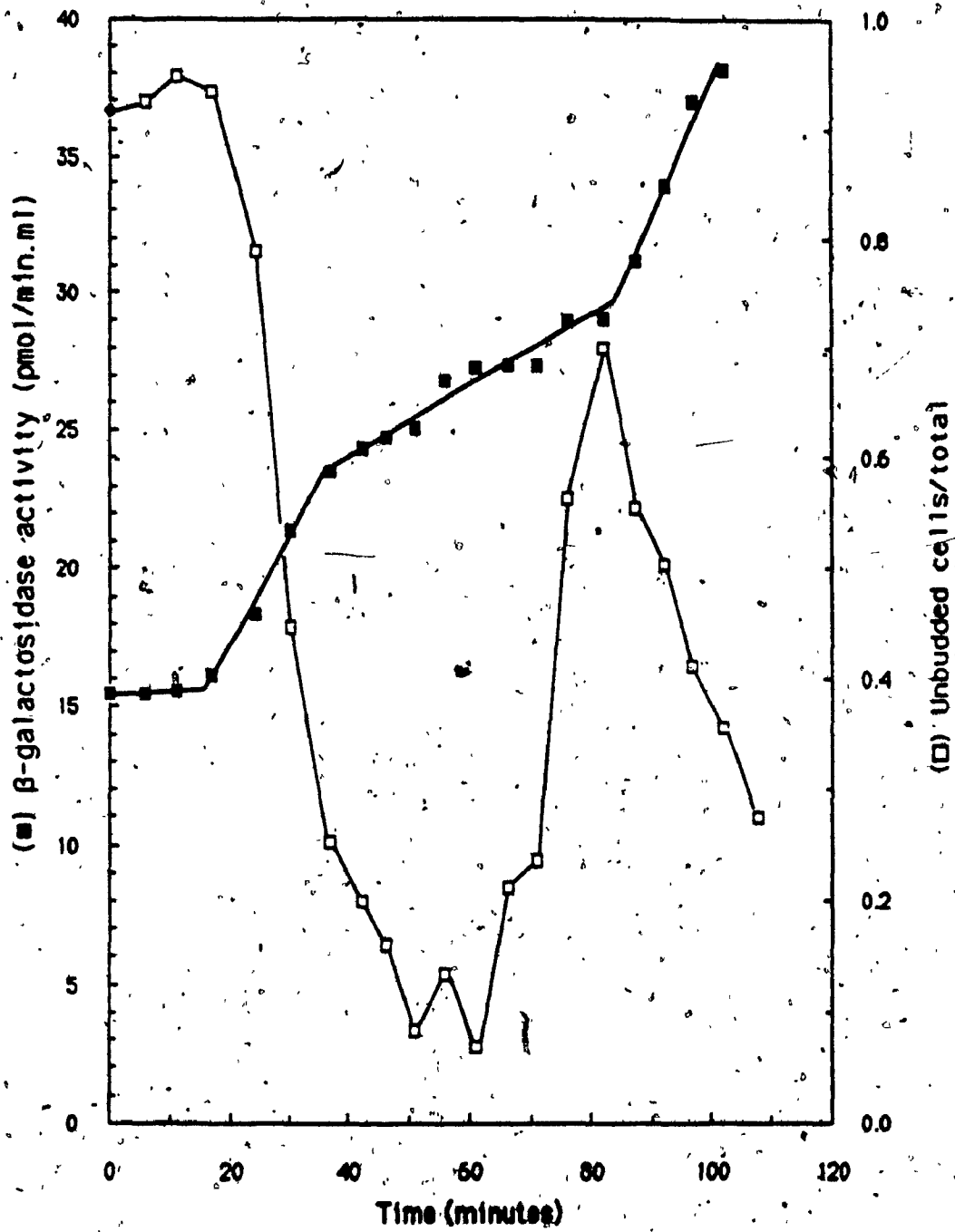


Figure 22. "Step" β gal activity profile during the cell cycle from pRS665 (an integrating version of pRS535) integrated in the yeast genome at leu2 in AH22 cells. Duplicate samples were assayed for β gal activity from each time point using $N_2(l)/BrI$ permeabilization. Sample ODs did not deviate from the means shown by more than 3%.

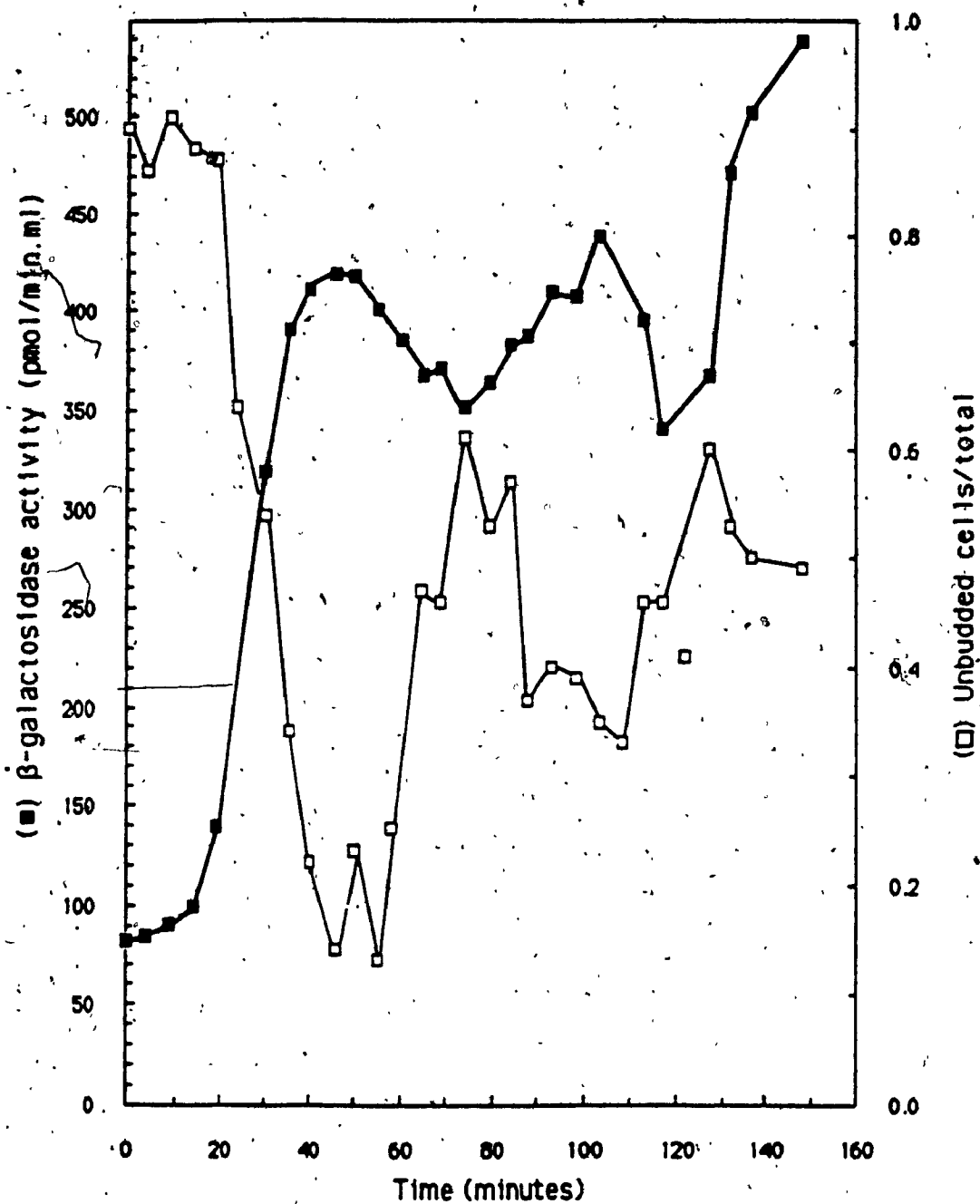


Integrand was lower due to the lower plasmid copy number (roughly 1/10, based on Table 3). Hence, this result also shows that low activities from this plasmid yield the same periodic profile as high activities, which is important to note for the mapping studies reported below.

C.3.3.e. Stable vs unstable Bgal activities. Because the periodic Bgal profile obtained from pRS535 shown in Figure 21 matched that predicted from total synthesized IMP1 RNA (Figure 18), it was of interest to compare the unstable Bgal activity produced from the pRS269 transformant with the IMP1 RNA "peak" profile also shown in Figure 18. The results shown in Figure 23, monitored over two generations show that the Bgal activity per ml of the fusion protein from pRS269 increases and declines periodically, and that the relative increases of the two generations correspond closely to the relative peak heights observed for IMP1 RNA (Figure 18). These results support the contention that the accumulation of fusion gene product reflects IMP1 mRNA synthesis. Furthermore, the peaks of activity suggest that the periodic profile of the pRS269 fusion product is caused by post-translational instability.

The failure of the second peak in Bgal activity accumulation per ml from pRS269 to surpass the first in size corresponds to the smaller peak of IMP1 RNA seen at this time (Figure 18). These smaller relative increases during the second generation are peculiar, since cell number has nearly doubled. Taken together, the data again suggest that a START-dependent induction may be associated with IMP1 RNA levels, or that the IMP1 gene from a sub-population of cells is not expressed.

Figure 23. "Peak" Bgal activity profile from pRS269, containing the intact 5' regulatory region, plus 2/5 of the coding sequence of TMP-1, fused to lacZ, in AH22, during two cell cycles, of synchronous growth. Duplicate samples were assayed for Bgal activity from each time point using chloroform/SDS permeabilization. Sample OD420s did not deviate from the means shown by more than 4%.



C.4. Mapping the IMP1 Regulatory Region using pRS535 Derivatives.

The above results, showing the periodic appearance of a stable Bgal activity from the IMP1'-lacZ fusion in pRS535-AH22, indicate that this fusion gene is cell cycle regulated. The control of this regulation probably stems from a sequence or sequences present in the 5' IMP1 fragment on the plasmid, since the promoting activity from pYT760-RYP3 (Figure 19) was non-periodic, and since a chromosomally integrated version of the plasmid retained the periodic behavior (Figure 22). The combined synchronization/Bgal assay procedure therefore permitted mapping of the 432 bp HindIII - Sau3A IMP1 5' region for the sequences responsible for this phenotype, by comparing the Bgal activity expressed from IMP1'-lacZ fusions deleted for sequences in this region, with pRS535-AH22. In order to map regulatory sequences, both the Bgal activities from batch cultures, and activity profiles during synchronous growth from the pRS535 mutant derivatives described in Figure 11 were compared with those from pRS535. The predicted phenotype for a IMP1'-lacZ fusion which had lost cell cycle regulation was a non-periodic activity profile such as those shown from pYT760-RYP3-AH22 (Figure 19), or possibly from pLG669-Z in DBY747 (Figure 20).

C.4.1. B-galactosidase Accumulation in Batch Cultures.

Based on the behavior of the pRS535 gene product in yeast, it was reasonable to assume that regulatory mutations present in the pRS535 derivatives could affect levels of Bgal expression in addition to affecting periodic expression. To test this possibility, plasmids derived from pRS535 whose 5' regulatory region had been altered were tested for their ability to express Bgal activity in yeast. Levels of Bgal activity were determined for batch cultures, during logarithmic growth (Tables

TABLE 4a Activities from TMP1'-lacZ fusions containing different TMP1' 5' ends




Plasmid	Description	Average β gal Activity / OD600		
In A122		Log phase	Stationary phase	Ratio
PRS535	Intact or		1160	1.8
PRS669	Δ Hind-Pst		1050	1.6
pTL30			1083	1.7
PRS535-10C	Inverted Mlu fragment	1547	5500	3.6
pTL35	Δ Hind-5'Mlu	2549	7255	2.9
pTL31	Δ Hind-3'Mlu	28	27	1.0
PRS535-1A	Δ Mlu		119	1.5
pTL32	Δ Hind-Pst		118	1.6
PRS269	Intact or		299	0.25
PRS676	Δ Hind-Pst		236	0.18
PRS535-7A			278	0.17
PRS264	Out-of frame fusion	12	15	1.3

TABLE 4B: Log phase β -gal activities in AH22 derivatives bearing pRS535-type plasmids.

Plasmid in yeast strain AH22	Region deleted	Average β gal Activity		
		per OD600	per mg. protein	per 10E7 cells
pRS535	none	635	2455	144
pTL30	Δ HindIII-PstI	678	2362	154
pRS669	Δ HindIII-PstI	655	2343	149
p535-10C	Region 3 inverted	1547	5462	351
pTL35	Δ HindIII - 5' MluI site	2549	5896	579
pTL31	Δ HindIII - 3' MluI site	28	79	6
p535-1A	Δ Mlu	79	334	18
pTL32	Δ Mlu; Δ HindIII-PstI	76	198	17
pYT760-RYP3	Intact RYP3	658	2556	149
pRS269	None; unstable β gal activities; extra 330 bp of coding DNA.	1162	4032	264
pRS676		1300	4285	295
p535-7A		1664	5083	378
pRS264	Out-of-frame fusion	12	46	3

Cell numbers ($\times 10^7$)/ml were 10.9 ± 2.0 (SD) in stationary (Stat) phase, 1.8 ± 0.4 in log phase and 1.2 ± 0.3 after 1.5 hr in alpha factor medium.

The average OD600/ml was 3.75 ± 0.27 (STAT), 0.48 ± 0.07 (LOG) and 0.34 ± 0.02 (ALPHA).

The mean mgs protein/ml (\pm SD) were, respectively, 0.98 ± 0.08 , 0.12 ± 0.02 and 0.68 ± 0.026 ($n = 12$ cultures).

Values per sample are the average of two determinations.

4A and B), stationary phase (Table 4A), and during arrest with the α -factor mating pheromone (see Table 5). Cells were grown in 2 x YNB medium with selection for leucine prototrophy.

Table 4A shows typical β gal activities from log phase transformants containing pRS535 and the derivatives. Strains showing similar β gal phenotypes have been grouped together. The results shown in Table 4A indicate that several of the alterations created in the HindIII - Sau3A region of IMP1 led to altered levels of β gal expression from the derived fusion gene in AH22, when compared with that from the parent plasmid, pRS535. However, the HindIII - PstI region of IMP1 was not essential for the expression of activity, since pTL30-AH22 and pRS669-AH22, which harbour plasmids with this region deleted (see also Figure 11) expressed β gal activity at levels very similar to pRS535 itself.

In contrast to the HindIII - PstI deletions, the presumptive deletion from the HindIII up to the 5' MluI site in pTL35 resulted in a four-fold increase in log phase β gal activity, which suggested that sequences located 5' to the upstream MluI site may not be required for β gal activity associated with pRS535, but may instead function in a negative fashion.

The deletion derivative missing information from the HindIII to the 3' MluI site, pTL31, resulted in a 25-fold reduction in β gal expression compared with that from pRS535. This result therefore suggested that the region between the two MluI sites contained a regulatory region required for high levels of expression. This interpretation was supported by the β gal phenotype from the last unique deletion, pRS535-1A, from which only the 37 base pair region between the MluI sites had been removed. β gal activity from this plasmid was reduced eight-fold (Table 4).

The pRS535-10C derivative bearing an inverted MluI region

expressed Bgal activity at levels 2.4-fold higher than pRS535-AH22. In the light of the above observations, this observation suggests that the positive element existing at or between the MluI sites functions independently of its orientation, but that one orientation results in higher expression.

In addition to pRS535 derivatives, several pRS269-type transformants and a pRS264 transformant were tested in this experiment. As before, activity from the pRS264 transformant was minimal, comparable with that of the HindIII-3'MluI deletion plasmid pTL31. Both the pRS269-type plasmid pRS535-7A described in the "Constructions" section, and pRS676, a pRS535 derivative lacking the small PstI fragment (see Figures 6 and 11), show the higher log phase activities and lower stationary phase activities described by Greenwood. Taken together, the similar behavior expressed from these independent constructions, as well as the similar behavior in yeast of pRS535, pTL30 and pRS669, tend to confirm the pRS269-AH22 and pRS535-AH22 phenotypes discussed earlier.

The diagnostic Bgal activity levels for a given lacZ transformant in yeast were also monitored with cells in stationary phase (G_0) cultures (Table 4A). Cells grown for two days in any of the media employed would have entered stationary phase by this time, and so cultures have roughly equal cell densities for purposes of comparison. Table 4A therefore also shows the G_0 Bgal activities observed from the same plasmids described in the previous section. The different constructs produce Bgal values which vary in a manner similar to those seen for log phase cells. For example, once again it can be seen that information 5' to the PstI site (Figure 3) has no effect on the levels of gene product expression.

Interestingly, the control plasmid pYT760 -RYP3 and plasmids

pRS535-10C and pTL35 confer higher G_0/\log activity ratios (2.9-, 2.9- and 3.6-fold, respectively), compared with the G_0/\log activity ratios from pRS535 (a ratio of 1.8-fold). Although the ratios observed here were significantly different, the possibility of " G_0 control" was not investigated further.

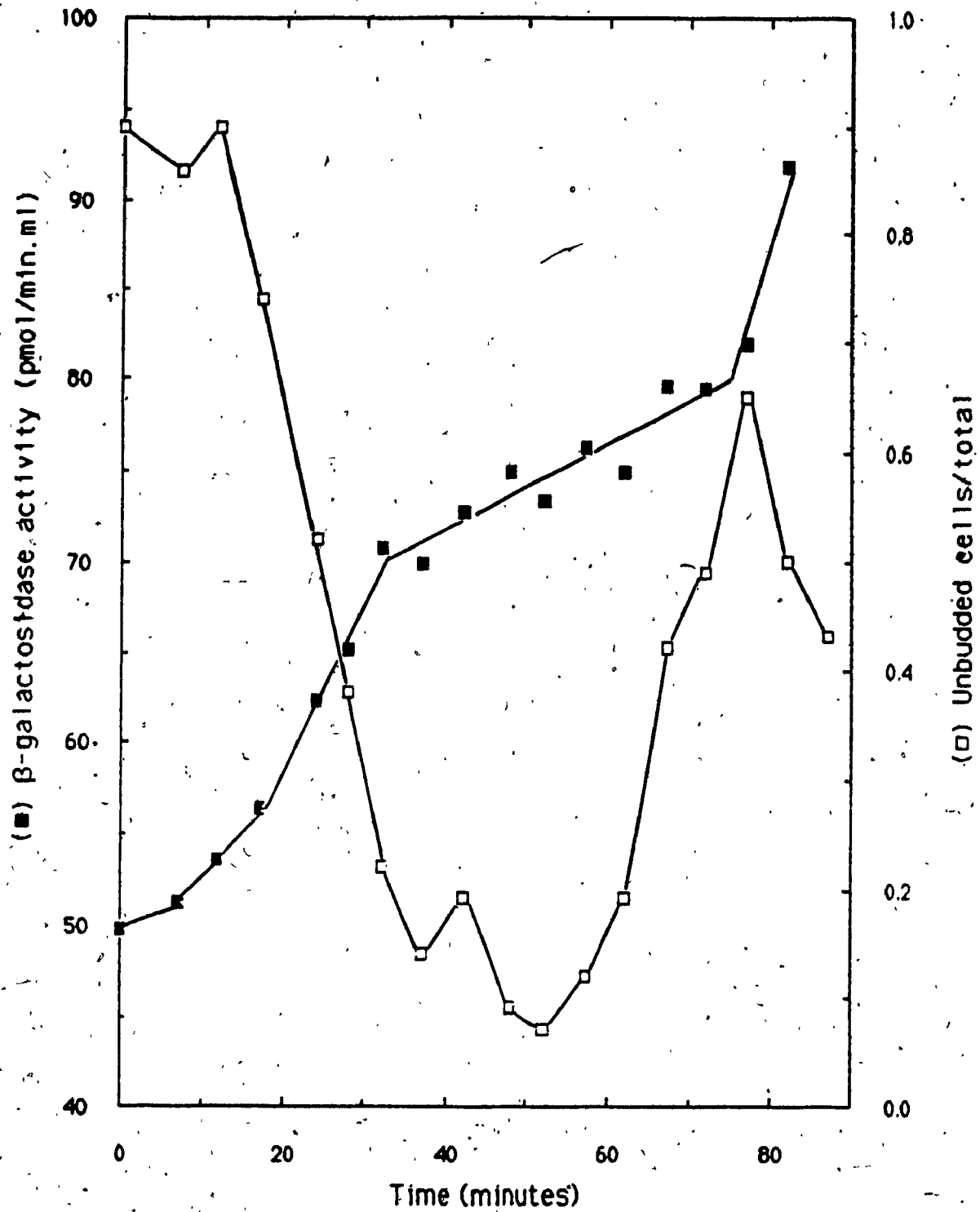
It should be noted that the removal of the HindIII to PstI fragment as well as the MluI fragment (in the derivative pTL32; Figure 11, Table 4A) has no detectable effect other than that due to the deletion of the MluI fragment alone. This further supports the supposition that the 5' HindIII to PstI fragment is not involved in the types of regulation described here, and supports the MluI deletion phenotype.

Because it was possible that log phase activities from the different plasmids varied because of differences in cell size, the log phase activities per mg protein, per 10^7 cells and per OD600 were directly compared in Table 4B, for the same experiment shown in Table 4A. As can be seen, the differences between the grouped plasmids remained the same regardless of whether activity was measured per cell, per OD600 or per mg. protein.

C.4.2. β -galactosidase Accumulation in Synchronized Cells bearing pRS535 derivative plasmids.

C.4.2.a. pTL30/pRS669 (Δ HindIII-PstI) The results of the above characterization encouraged me to monitor the β gal activity of some of the pRS535-AH22 derivatives during synchronized growth. Because deletion of the HindIII to PstI fragment did not affect any of the pRS535-AH22 β gal phenotypes (Tables 4a and b), a synchrony of AH22-pRS669 bearing the HindIII-PstI deletion was monitored for its β gal activity profile (Figure 24). The observed periodic β gal activity

Figure 24. "Step" Bgl activity profile from pRS669, containing a 5' IMP1 regulatory region deleted only for the upstream information between HindIII and PstI (see Figure 11) fused to lacZ, in AH22, during one cell cycle of synchronous growth. Duplicate samples were assayed for Bgl activity from each time point using $N_2(1)/Brij$ permeabilization, with different assay incubation times for each of the pair (125 vs 425 minutes). After correcting for incubation time, activities did not deviate from the means shown by more than 3%.



profile mimicked that from pRS535, and supports the conclusion drawn from Table 4 - that the HindIII to PstI region is not essential for the types of regulation studied here.

C.4.2.b. pRS535-1A. (Δ Mlu)

Next, *Bgal* expression from pRS535-1A, deleted only for the MluI fragment, was monitored in yeast during synchronous growth. The particular isolate used for this experiment showed poor cytokinesis (bud separation) at the end of the first synchronous generation, and so the appearance of small buds (per total CFU) was monitored as an indicator of DNA synthesis initiation (Williamson and Scopes, 1961), instead of the disappearance of unbudded cells. As shown in Figure 25, the *Bgal* activity profile during synchronous growth was quite different from that observed for pRS535. Apart from a small induction after time zero, the activity accumulation was linear for over two generations of synchronous growth.

The pattern seen in Figure 25 suggested that pRS535-1A lacked cell cycle dependent regulation. However, the profile observed might have been generated by either of the non-periodic profiles predicted (Figures 19 and 20). In particular, a small induction of *Bgal* activity was observed after time zero in Figure 25, and so it was possible that the cell cycle regulation had been lost but that some form of START-inducible or proliferation-dependent control was still active. However, this effect might have been related to a defect in the strain, including the observed aberrant cytokinesis. As a result, a second isolate showing essentially normal cytokinesis was monitored during synchronous growth (Figure 26), and here the pattern was essentially the same as that seen for Figure 25; namely, an almost "continuous-linear" *Bgal* activity profile in

Figure 25. β gal activity profile from pRS53541A, which contains a IMP1 5' regulatory region deleted for the MluI region (Figures 3 and 1-1), fused to lacZ, in AH22, during two cell cycles of synchronous growth. Duplicate samples were assayed for β gal activity from each time point using $N_2(1)/Brij$ permeabilization. Sample OD420s did not deviate from the means shown by more than 4%, except at $t=25$ (8%). Note that newly budded cells per CFU are plotted instead of unbudded cells per CFU, as a cell cycle indicator, because of delayed cytokinesis in this strain.

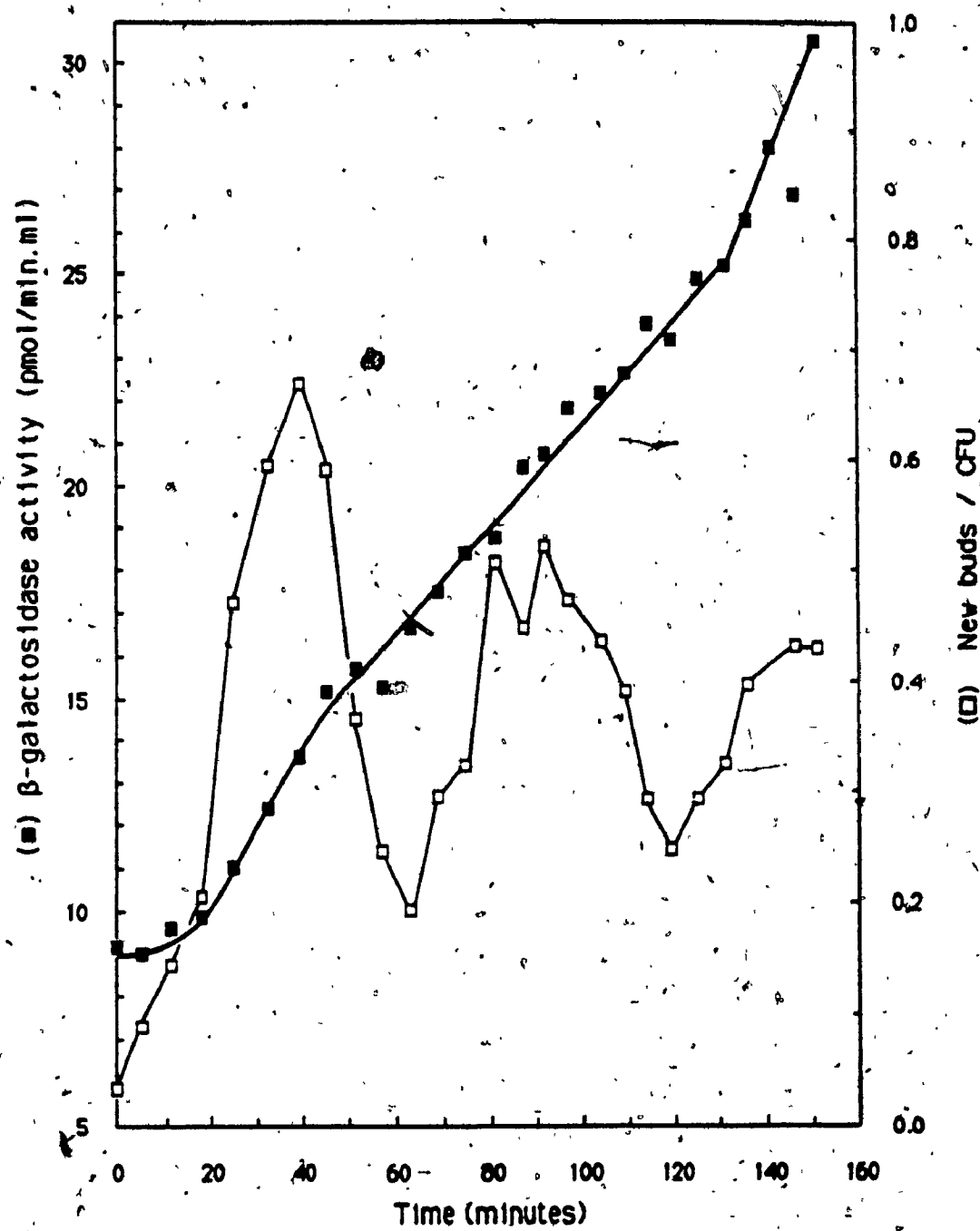


Figure 26. β gal activity profile from pRS535-1A, which is deleted for the MluI region, fused to lacZ, in a different AH22 strain from that portrayed in Figure 25, during one cell cycle of synchronous growth. Duplicate samples were assayed for β gal activity from each time point using $N_2(1)$ /Brij permeabilization. Sample OD420s did not deviate from the means shown by more than 3%.

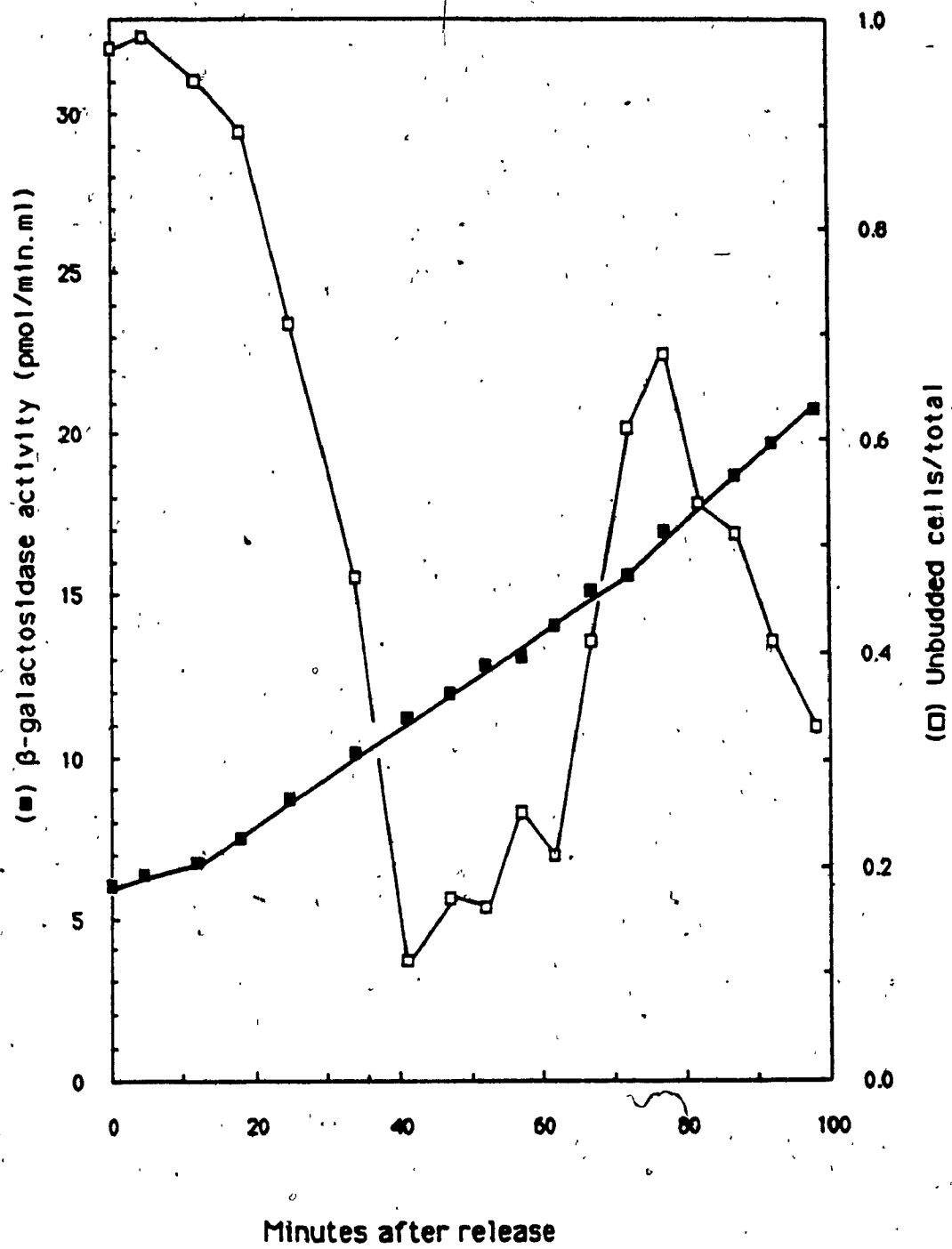
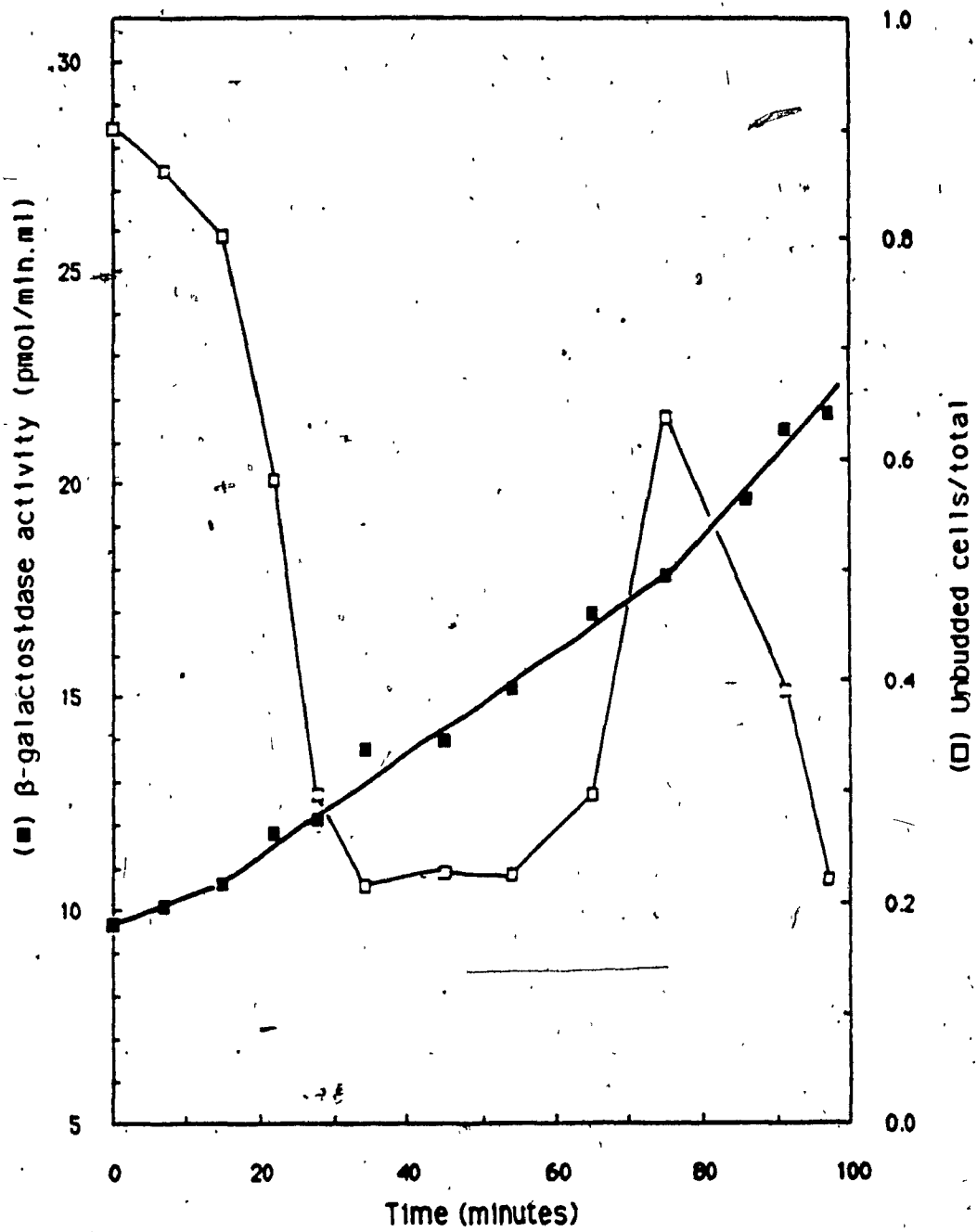


Figure 27. Bgal activity profile from pTL32, which contains the IMP1 5' regulatory region deleted for both the MluI region and the HindIII to PstI region (see Figure 11), fused to lacZ, in AH22, during one cell cycle of synchronous growth. Duplicate samples (shown) were assayed for Bgal activity from each time point using $N_2(1)$ /Brij permeabilization. Sample OD420s did not deviate from the mean values by more than 4%.



each generation. In this latter experiment, the activity profile approximated more closely that seen from pYT760-RYP3 (Figure 19). By comparing Figures 25 and 26 with the results from plasmids bearing intact IMP1 MluI fragments (Figures 21 and 22) it was inferred that removal of the MluI fragment had considerably reduced periodic expression from the IMP1-lacZ fusion gene. This inference was supported by the observation that the pRS535 derivative pTL32, deleted for both the 5' HindIII - PstI sequence and the MluI fragment, yielded essentially the same, non-cell-cycle regulated profile (Figure 27) as that seen for pRS535-1A-AH22 (Figure 26). This result suggested that the MluI sequence was required for periodic expression, and that the HindIII - PstI region had no additional effect.

C.4.2.c. pTL35 (Δ HindIII - 5' MluI)

The pTL35 derivative in AH22 was also monitored during synchronous growth, to determine whether the presence of the MluI fragment alone was sufficient to periodically express the derived gene fusion. This appeared to be the case, since activity/ml increased in a periodic fashion when monitored through one cell cycle (Figure 28). This result again suggests that the MluI fragment is required for periodic expression.

C.4.2.d. pRS535-10C. (inverted MluI)

The above results, combined with the data from Table 4a, suggest that the MluI fragment is required to maintain high levels of gene product, as well as to periodically express the pRS535 IMP1-lacZ gene. It was therefore of interest to determine whether the pRS535-10C fusion containing a 5' IMP1 regulatory region with the MluI fragment inverted (Figure 11) was periodically expressed, since the inversion did not result

Figure 28. β gal activity profile from pTL35, which purportedly contains no IMP1 DNA 5' to the 37 bp Mlu1 region (see Figures 3 and 11), with the rest fused to 'lacZ', in AH22, during one cell cycle of synchronous growth. Samples were assayed for β gal activity from each time point using $N_2(1)$ /Brij permeabilization.

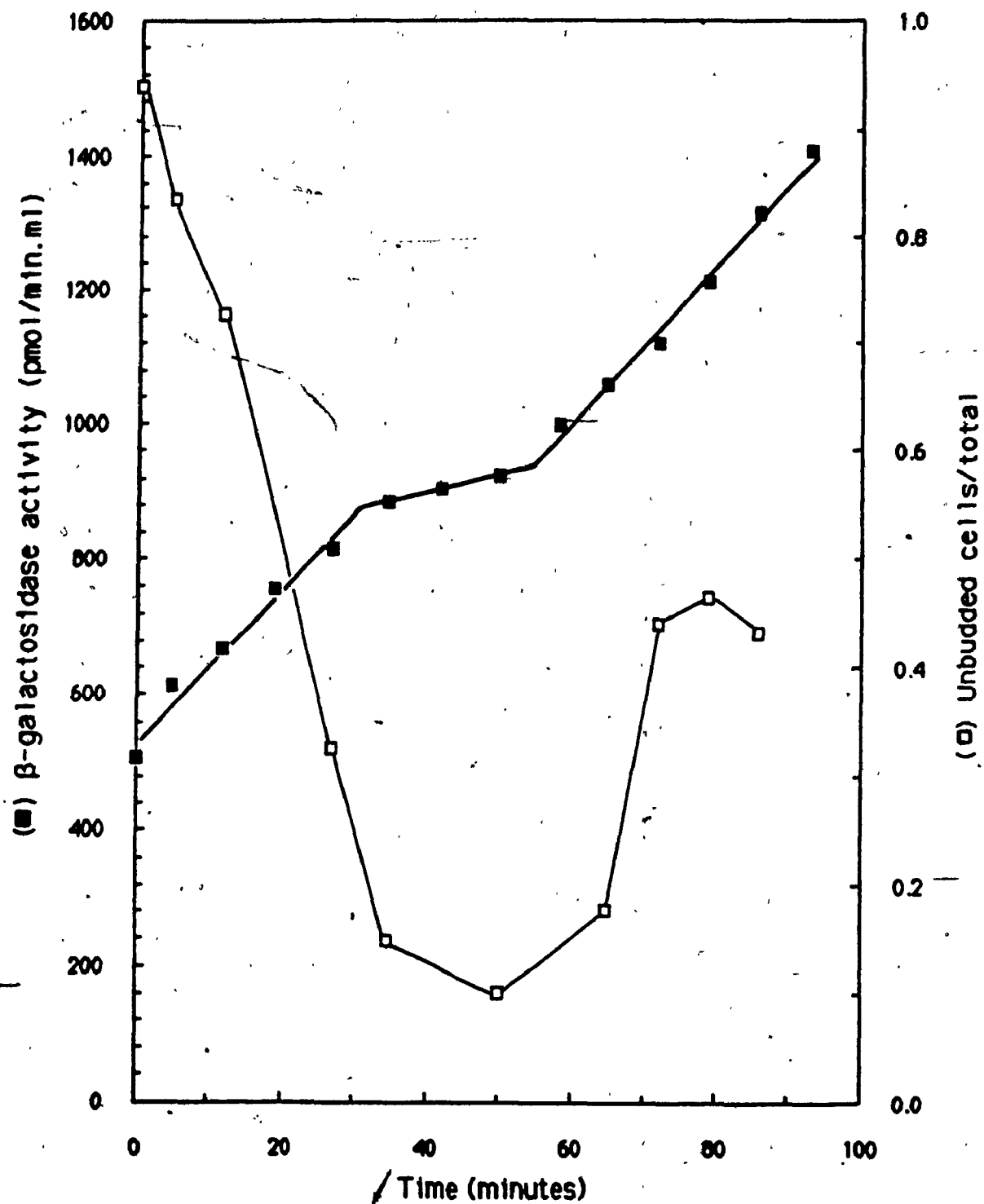
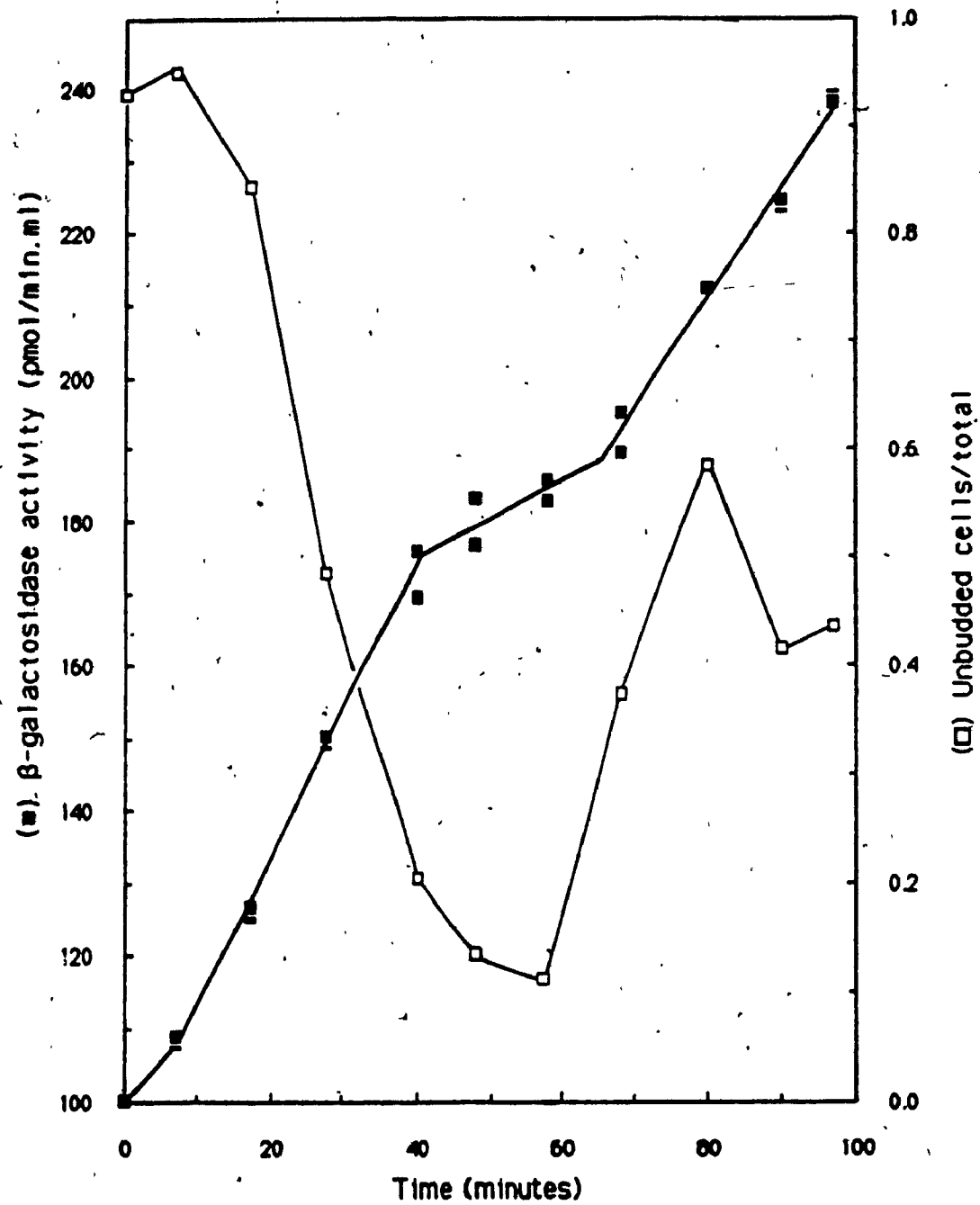


Figure 29. *Bgl*I activity profile from pRS535-10C, which contains a IMP1 5' regulatory region with an inverted, MluI region (see Figure 3), fused to 'lacZ, in AH22, during one cell cycle of synchronous growth. Duplicate samples (shown in the Figure) were assayed for *Bgl*I activity from each time point using N₂(1)/Brij permeabilization.



in the loss of Bgal activity. The results shown in Figure 29 suggest that this is the case, in that a step profile is observed from synchronized pRS535-10C/AH22 cells. Therefore the MluI sequence may comprise part or all of a major, orientation independent TMP1 5' regulatory element.

C.5. B-galactosidase Accumulation in α -arrested cells.

Nasmyth (1983) has shown that cells do not express HQ RNA or gene product at the G1 stage of the cell cycle. The yeast pheromone α -factor used to synchronize cells for both Nasmyth's data and for the above experiments arrests cells in G1, and the synchronous profiles noted from the periodically expressed TMP1'-lacZ gene fusions strongly suggest that the pheromone reduced Bgal activity from these constructions in G1, since accumulation increases following release. In a preliminary experiment performed at the same time as that shown in Table 4, Bgal activity accumulations from the non-periodically expressed strains bearing pYT760-RYP3, pRS535-1A, pTL32 or pRS264 were not reduced in the presence of α -factor (compared with log phase cells). In contrast, Bgal activity accumulations in the periodically expressed strains noted above were curtailed in α -factor (see Table 5 below). To confirm that the MluI-deleted pRS535-AH22 derivatives had lost a form of down-regulation seen from pRS535-AH22 during α -factor arrest, cells growing asynchronously in 2xYNB medium were arrested with α -factor, and Bgal activity was monitored as cells were arresting. The results (Figures 30, 32 and 33) are plotted on log scales to interpolate the timing of any down-regulation of Bgal activity during α -factor arrest.

Accumulation of Bgal activity from pRS535-AH22 declined due to α -factor, relative to untreated cells, and the slope of the α -factor regulated profile interpolated back to the time of pheromone addition

Figure 30. Requirement of the MluI fragment for down-regulation of β gal activity from pRS535 during α -factor arrest. Activity increases are plotted logarithmically (left-hand axis), while changes in budding activity are plotted linearly (right-hand axis).

A & B. Immediately divergent β gal activity profile during α -arrest from pRS535-AH22, compared with the β gal profile of the same culture without α -factor. (A) At $t=0$ one half of a log phase pRS535-AH22 culture (in 2 x YNBH+D) was mixed 1:1 with either α -factor medium, and the other half with medium lacking α -factor. To make the latter more similar to the α -factor medium, X2180-1A, a non- α -factor-producing strain, was grown for the same 48 hour period in the same 1 x YNBD medium as the S288C α -factor producing strain. The timing of the α -arrest is depicted by the decrease in proportion of new buds (small buds or SB) per colony forming unit (SB/CFU). Duplicate samples were assayed for β gal activity from each time point using $N_2(1)/Brij$ permeabilization. Sample OD420s did not deviate from the means shown by more than 5%. (B) Same experiment as "A", except 100 μ g of synthetic α -factor in 100 μ l of water was added to a 100 ml culture, while a parallel culture was not tampered with. Duplicate activities per sample did not deviate from the means shown by more than 3%.

C & D. Tangentially-divergent β gal activity profile during α -arrest from pRS535-1A-AH22 deleted for the 5' IMP1 MluI fragment, compared with the same culture without α -factor. (C) Conditions were identical to those shown in Figure 30A. (D) Conditions were as given for Figure 30B, except a 120 ml culture was used. Separate sample activities did not deviate from the means shown by more than 5%.

E and F. Non-divergent β gal activity profile during α -arrest from pYT760-RYP3, compared with the same culture without α -factor. (E) Conditions were identical to those shown in Figure 30A. (F) Conditions were as given for Figure 30B, except a 150 ml culture was used. Sample OD420s did not deviate from the means shown by more than 5%.

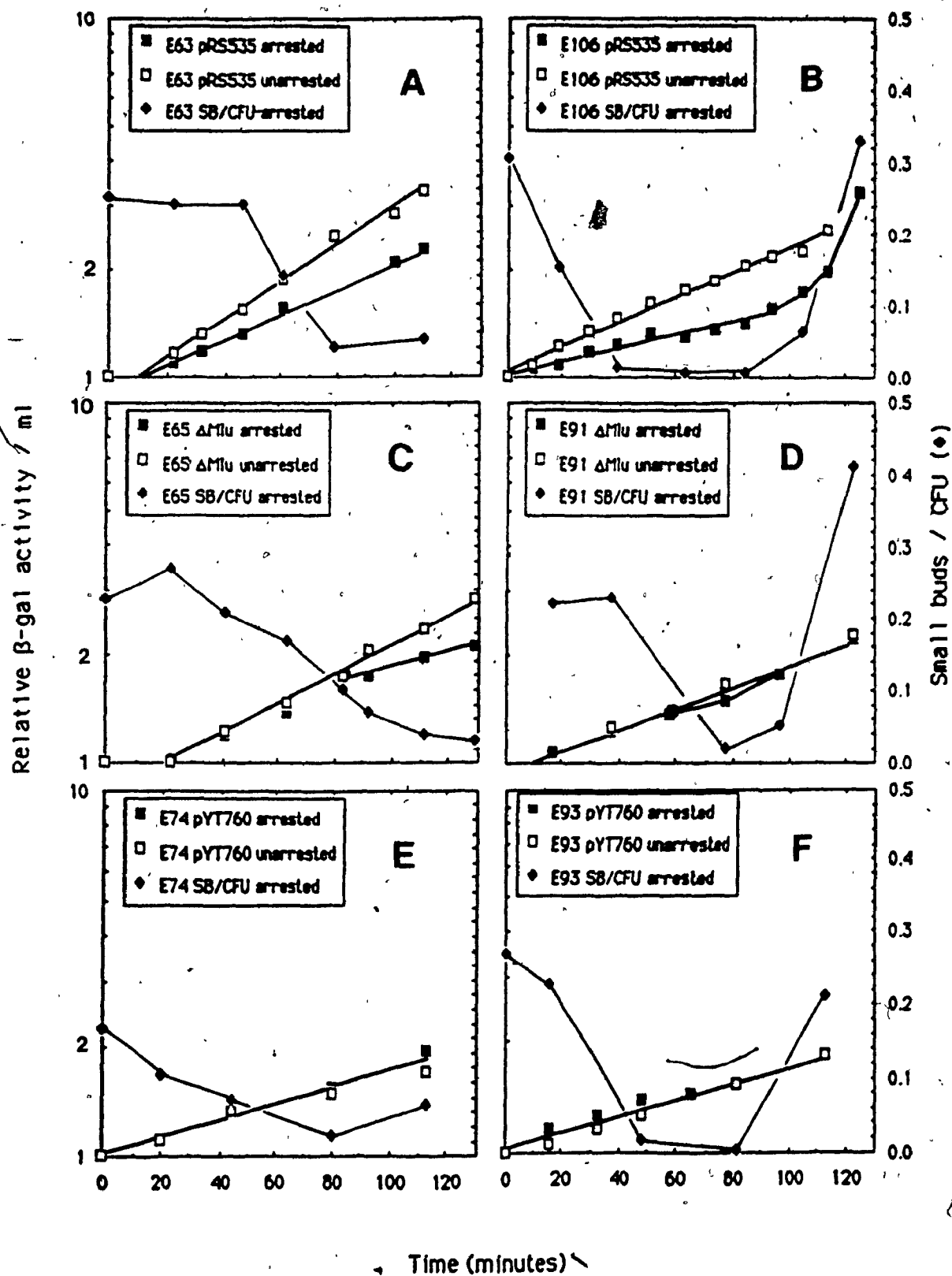


Figure 31. Requirement of the MIU1 fragment for down-regulation of *Bgal* activity from pRS535 during α -factor arrest. Data is the same as that shown in Figure 30 except that activity increases are plotted linearly (left-hand axis).

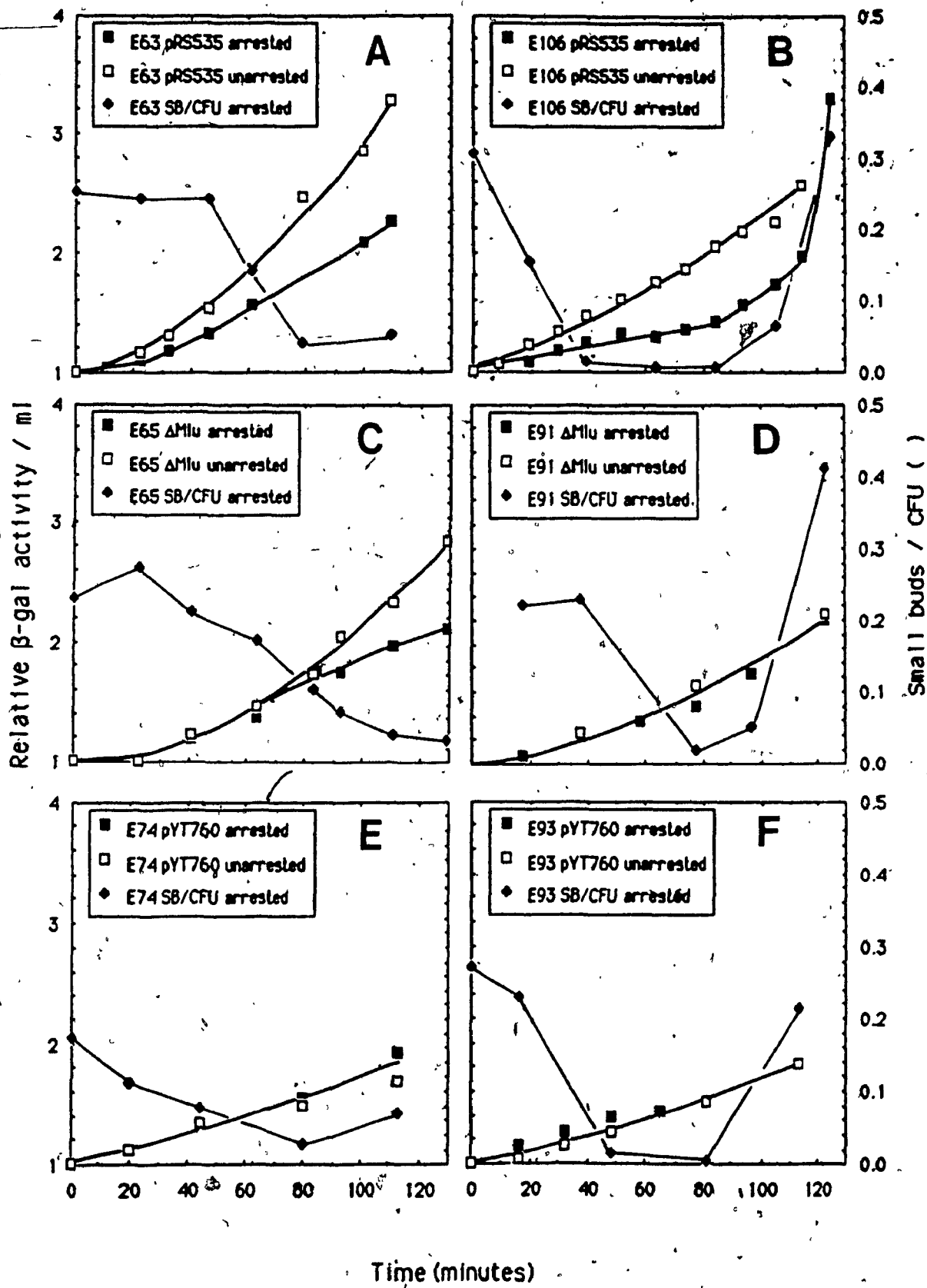


Figure 32. *Bgal* activity profile before and during α -arrest, and after recovery from α -arrest, from pRS535-1A, which lacks the IMP1 MluI fragment, compared with the same culture without α -factor. This data is from the same experiment shown in Figure 30D, except that values before and after α -arrest are shown. Assay conditions were identical to those shown in Figure 30A. Separate sample activities did not deviate from the means shown by more than 5%.

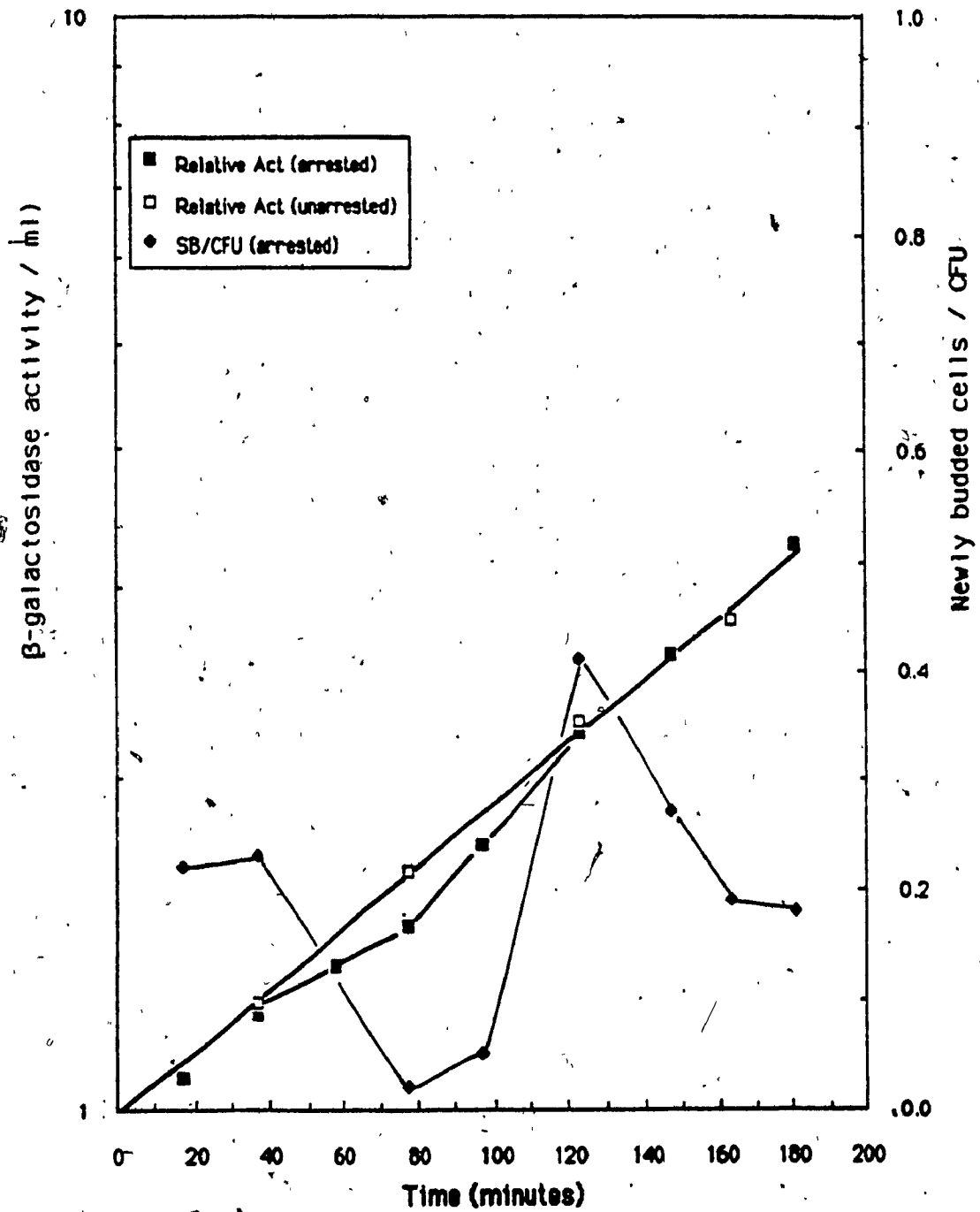
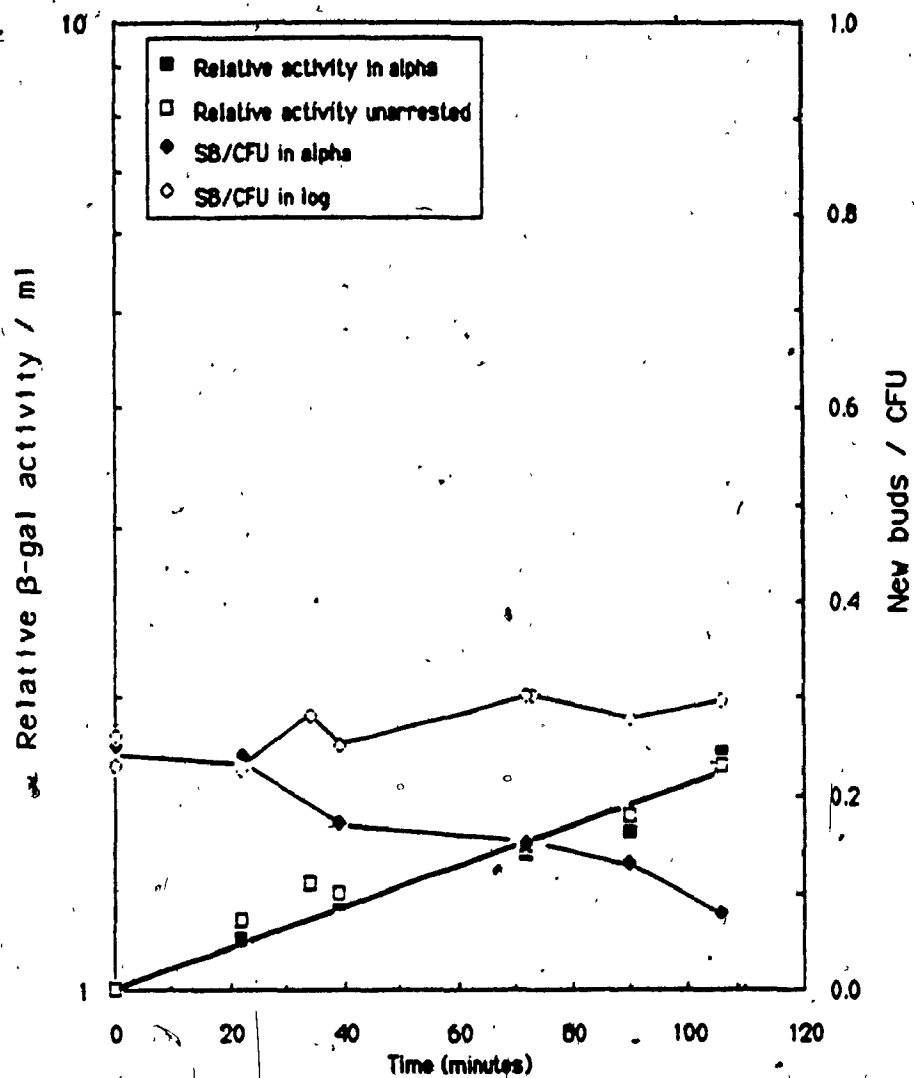


Figure 33. Non-divergent Bgal activity profile during α -arrest from pTL31-AH22 deleted for all information 5' to the downstream IMP1, MluI site (Figure 11), compared with the same culture mixed with X2180-1A - conditioned medium without α -factor. The proportion of newly-budded cells in the unarrested culture was included for comparison. Conditions were otherwise identical to those shown in Figure 30A. Sample activities did not deviate from the means shown by more than 3%.



(Figures 30 and 31; A and B). This result contrasts with those observed from pRS535-1A (ibid; plots C and D) or pYT760-RYP3 (ibid; plots E and F), whose activities during arrest showed little change from that of the asynchronous culture, until the cells had arrested due to the pheromone. In other words, the slope of the activity curve in the non-periodically expressed strains during α -factor arrest only diverged tangentially from that of the parallel unarrested culture, and such a divergence can be attributed to reduced cell division as opposed to regulation.

Interestingly, the activity from the non-periodically expressed Δ MluI strain tended to return to a level of activity equivalent to the unarrested parallel culture (see Figure 32, where the complete results from the experiment shown in Figure 30D have been plotted). This compensating phenomenon, although repeated in other experiments (not shown), could be due to the similar timing of these α -factor arrests (≈ 120 min), which corresponds to a cell doubling time, and to the approximate doubling observed for β gal activity as the strain escapes from α -factor arrest. Although interesting, this compensating or "rebound" effect was not tested further during in this study.

The last pRS535 derivative tested for β gal expression in yeast during α -factor arrest was pTL31, which lacks all IMP1 sequences 5' to the downstream MluI site. The activity during arrest in this strain (Figure 33) was again not significantly different from that of the unarrested cells, although the activity was very low and therefore quite variable.

In summary, the results for α -factor arrested cells suggest that β gal expression from pRS535 is down-regulated immediately upon the addition of α -factor pheromone, and that a sequence in the 5' IMP1 region, which includes the same MluI fragment required for periodic expression, is

implicated in this down regulation.

C.6. IMP1 Versus Fusion-encoded total RNA Levels.

The observations described above provide evidence that the periodic expression of IMP1 is regulated by a sequence found within or overlapping the MluI fragment upstream of the structural gene. Although the element lies 5' to several putative transcriptional start signals and mapped mRNA 5' ends (E. McIntosh, pers. comm.), a determination of whether the gene is subjected to periodic pre- or post-transcriptional regulation by sequences in this region awaits another study. However, since the IMP1-specific RNA peaks observed by Storms *et al* (1985) were induced by release from α -factor arrest, it was tempting to speculate that RNA from the pRS535 fusion was periodically expressed, and that its transcription was curtailed in the presence of α -factor.

The final part of this project was to initiate studies of the transcriptional regulation of IMP1 by studying the regulation of total IMP1'-'lacZ RNA in yeast strain pRS535-AH22 and some of the strains carrying derivatives of pRS535. The first prediction was that IMP1'-'lacZ fusion RNA would be regulated similarly to IMP1 RNA; that is, fusion RNA levels would rise and fall periodically (see Figure 18), and would be reduced in the presence of α -factor (Storms *et al*, 1985). In addition, based on the *Bgl*I activity data, the RNA levels expressed by pRS535-1A (the derivative missing the MluI fragment) were expected to be significantly reduced in log phase cultures and unaffected by the addition of α -factor. On the other hand, RNA levels expressed from pRS535-10C-AH22 (this strain harbours the pRS535 derivative with the MluI-fragment-inverted) should be somewhat higher than that observed for pRS535-AH22, but still sensitive to the addition of α -factor.

C.6.1. Fusion RNA from Synchronized Cells. To test whether the IMP1'-lacZ fusion transcript was periodically expressed, total yeast RNA, isolated from synchronized cells bearing either pRS535, pRS269 or plasmid pRS306 [a plasmid similar to pRS665 (Figure 5A) but bearing the same amount of IMP1 coding DNA as pRS269], was Northern-blotted, and probed for the presence of fusion RNA (Figures 34, 36 and 37 respectively). In each of these experiments, the fusion 3.3 Kb RNA profile from the replicating plasmids did not closely resemble the periodic IMP1 RNA profile until the second synchronous generation, even though their respective fusion-directed β gal activity profiles were normal (see Figure 35, other β gal profiles not shown).

Figure 34A shows the IMP1 and IMP1'-lacZ RNA profiles from a synchronous pRS535-AH22 culture detected with a IMP1 HindIII - BamHI probe. Figure 34B shows the same blot probed with lacZ (BamHI - SstI) DNA to detect only the IMP1'-lacZ transcript. Figure 34C shows the genomic actin transcript (lower band) and a plasmid-derived pBR322 transcript (see below) detected by reprobing the same blot with the pYAct plasmid (donated by R. Ng, which contains actin and pBR322 DNA). As can be seen from Figure 34C, the levels of both actin and of the pBR322 transcripts were constant throughout the synchrony with the exception of lane 6, where the high values are probably due to a loading error. Therefore, these two RNA species can be used as non-periodic control RNAs. The pBR322 RNA was fortuitously transcribed from all the plasmids tested (eg. Osley and Hereford, 1981). Since this pBR322 transcript is from plasmid DNA, it also controls for plasmid copy number and for plasmid-related transcriptional artifacts. The pBR322 transcript was therefore used to correct quantitated lacZ RNA levels for loading or

Figure 34. RNAs expressed from pRS535 in yeast strain AH22, isolated from synchronized cells, fractionated on a 1.5% agarose gel, and Northern blotted to nylon (PALL-Biodyne) membranes. RNAs shown are from 25 ml aliquots sampled as follows: lane 1, asynchronous culture prior to α -factor addition; lane 2, cells arrested with α -factor sampled prior to release ($t=-20$ min); lane 3, first sample of synchronous cells at $t=0$ minutes after release; lane 4, 10 minutes; lane 5, 20 minutes; lane 6, 30 minutes; lane 7, 40 minutes; lane 8, 50 minutes; lane 9, 60 minutes; lane 10, 70 minutes; lane 11, 80 minutes; lane 12, 90 minutes. All lanes were loaded with 15 μ g of total yeast RNA. Note that lane 6 appears to be overloaded (see Appendix for sister-gel photographs).

- A. RNA (blot) probed with the HindIII-EcoRI fragment of TMP1 (see Figure 3; specific activity of the probe was $> 1 \times 10^7$ dpm/ μ g, exposure time was 5 days). The 3.3 Kb lacZ RNA and the TMP1 RNA band are indicated.
- B. The same RNA blot as A., probed with the BamHI-ClaI lacZ fragment from pMC1871 (specific activity of the probe was $> 1 \times 10^7$ dpm/ μ g, exposure time was four days). The 3.3 Kb lacZ-hybridizing RNA is indicated. The identity of the higher MW bands is not known.
- C. The same RNA blot as A., probed with the pYAct plasmid, (courtesy Ray Ng), which contains the yeast actin gene sequences, and pBR322 sequences homologous to a transcript from pBR322 DNA present in pRS535-type plasmids ("pBR322"). Specific activity of the probe was $> 1 \times 10^7$ dpm/ μ g, exposure time was 2 days. The lower actin RNA band shows some evidence of processing (seen as a double band in lanes 4, 5 and 6), which is expected for this intron containing gene. The identity of the higher MW bands is not known.

PROBE

173

BAND

TMP1

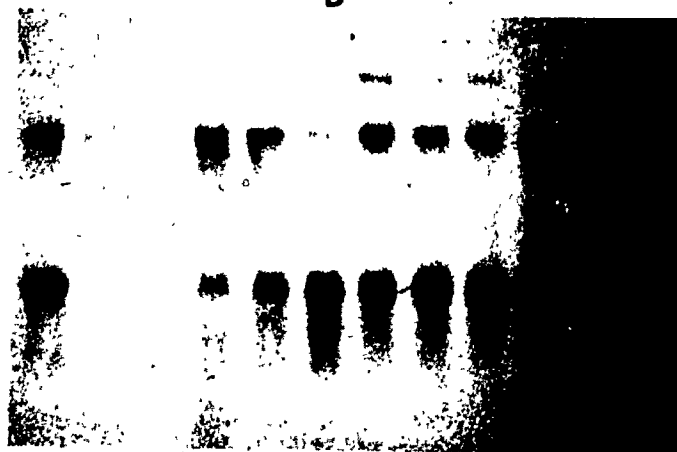
A



- — lacZ
- — TMP1

lacZ

B



- — lacZ

Actin /
pBR322

C



- — pBR322
- — Actin

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

Figure 35. Relative, densitometrically-quantitated RNA levels from synchronized. pRS535-AH22.

Top. Relative levels of IMP1 RNA (open squares), compared with the relative levels of IMP1-lacZ RNA (closed squares), during a synchronous cell cycle. The values, plotted relative to the arrested sample, were obtained by scanning a lighter exposure of the same probed blot shown in Figure 34A. Values were corrected for loading errors by dividing the scanned values by the scanned values obtained for the pBR322 transcript (Figure 34C).

Bottom. Relative Bgl activity accumulation and budding cycle from the same synchronized pRS535-AH22 culture used to isolate the RNAs shown in Figure 34 and in Figure 35 (top). Values are plotted relative to the t=0 sample.

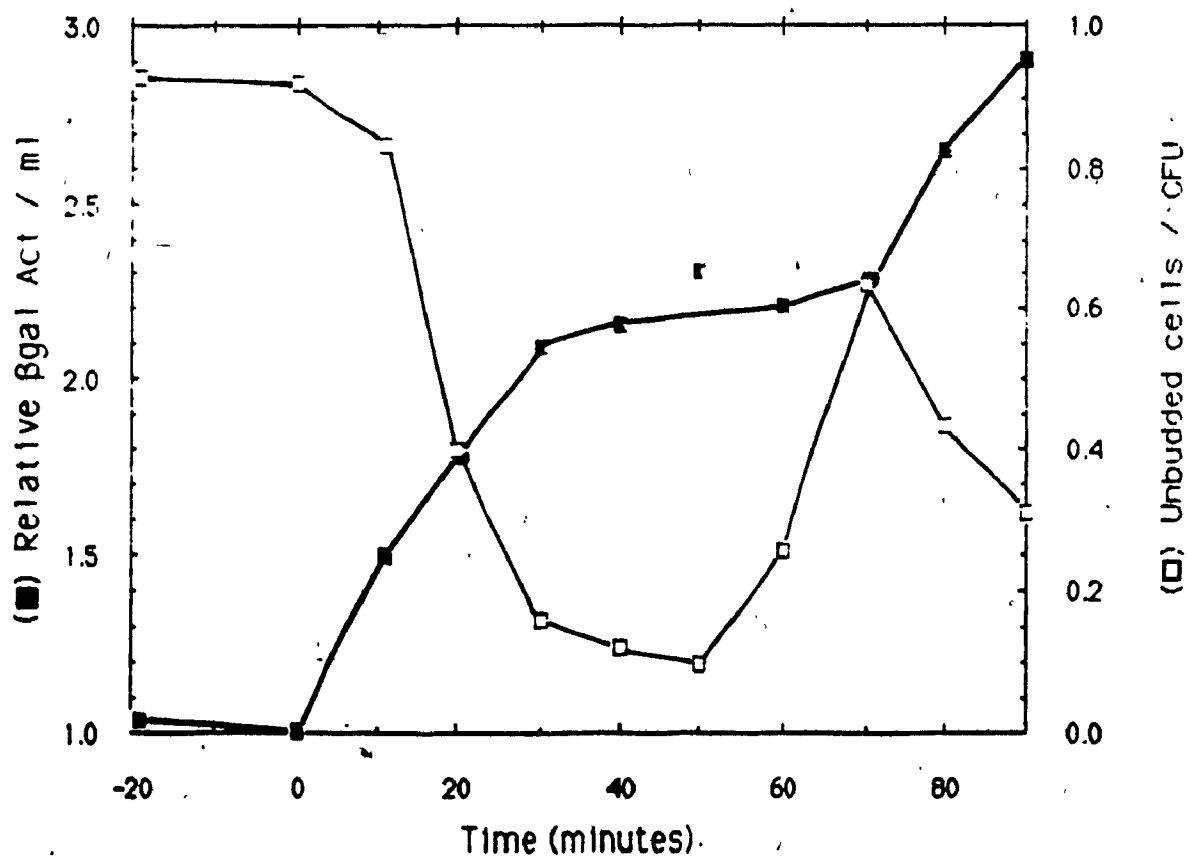
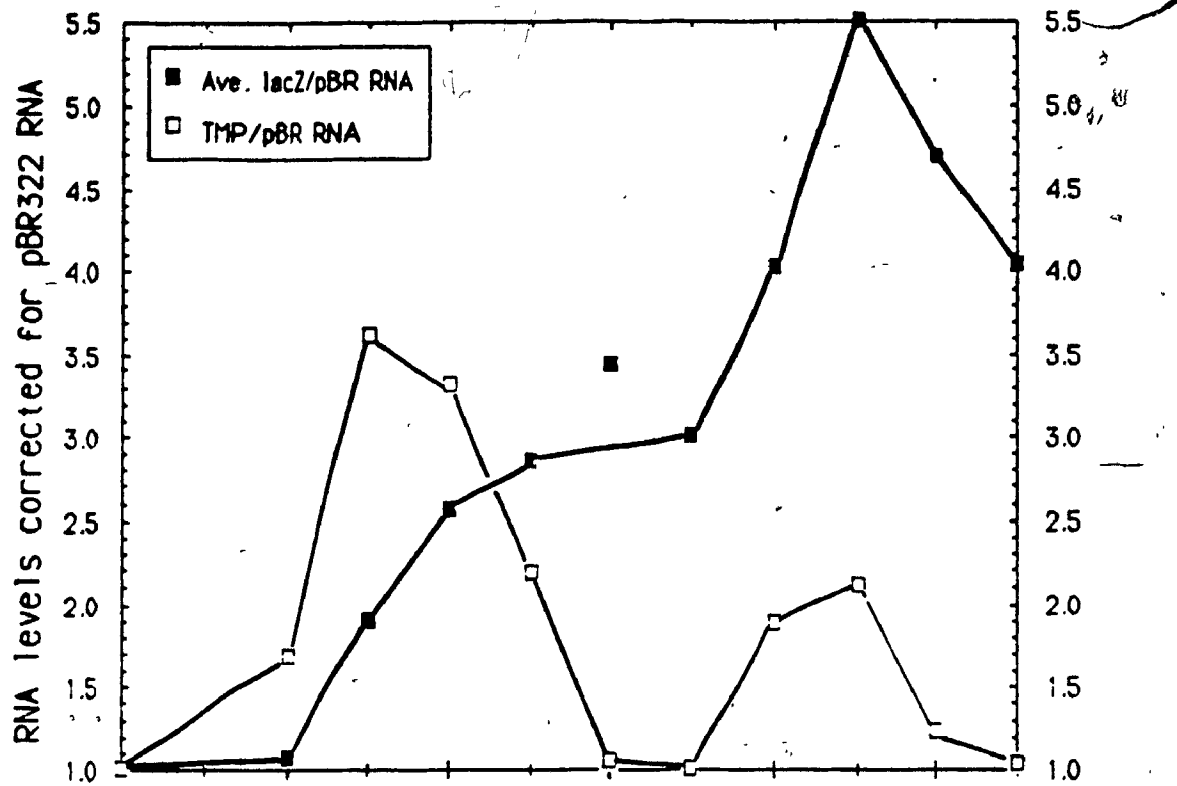


Figure 36. RNA expressed from pRS269-AH22, isolated from synchronized cells, fractionated on a 1% agarose gel and Northern blotted to a nylon (PAL-Biodyne) filter. The RNA blot was probed with the HindIII-BamHI IMP1 fragment (see Figure 3. Specific activity of the probe was $\approx 5 \times 10^7$ dpm/ μ g). RNAs shown are from 30 ml aliquots sampled as follows: lane 1, asynchronous culture prior to α -factor addition; lane 2, cells arrested with α -factor sampled prior to release ($t=-20$ min); lane 3, first sample of synchronous cells at $t=0$ minutes after release; lane 4, 10 minutes; lane 5, 20 minutes; lane 6, 30 minutes; lane 7, 40 minutes; lane 8, 50 minutes; lane 9, 60 minutes; lane 10, 70 minutes; lane 11, 80 minutes; lane 12, 90 minutes. All lanes were loaded with 13 μ g of total yeast RNA. Note that lanes 3 and 11 are underloaded (see Appendix for sister gel photographs). As a result, the second generation peak of both IMP1 RNA and of IMP1'-lacZ RNA likely occurs at 80 minutes.

- A. The 3.3 Kb IMP1'-lacZ RNA visualized after a short exposure (exposure time was 8 hours). The 3.3 Kb IMP1'-lacZ RNA band is indicated by an arrow. Note that the smear of hybridization seen at high MW in lanes 1, 2, 9, 10, and 12 may be due to degradation of pRS269 plasmid DNA, since the sister gel photographs showed a strongly staining, high MW band (see Appendix) which was also degraded in these lanes.
- B. Pattern observed after a deliberate overexposure to X-ray film of the same blot shown in A (exposure time was 4 days), in order to detect IMP1 RNAs. The IMP1 RNA band (the lowest band at the bottom of the gel) is indicated by the arrow. Note that the peak in IMP1 RNA levels at $t=20$ minutes (lane 5) and the broader peak in lanes 9-12.

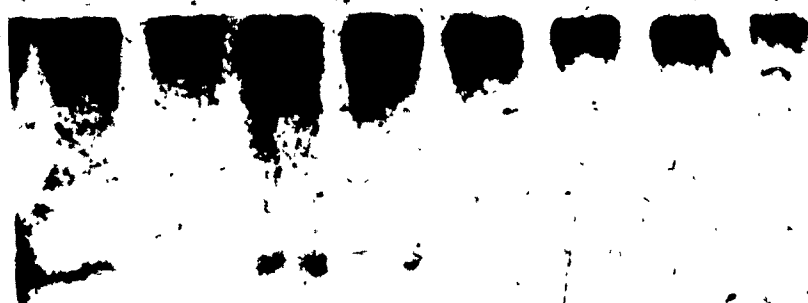
A

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

B

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

Figure 37. RNA expressed from pRS306-AH22, isolated from synchronized cells, fractionated on a 1% agarose gel, and Northern blotted to a nitrocellulose filter. The RNA (blot) was probed with the HindIII-BamHI region of IMP1 DNA (specific activity of the probe was $> 1 \times 10^7$ dpm/ μ g, and the blot was exposed to X-ray film for 7 days). RNAs shown are from 35 ml aliquotes sampled as follows: lane 1, first sample of synchronous cells at $t=0$ minutes after release; lane 2, 10 minutes; lane 3, 20 minutes; lane 4, 30 minutes; lane 5, 40 minutes; lane 6, 50 minutes; lane 7, 60 minutes; lane 8, 70 minutes; lane 9, 80 minutes. The bottom arrow shows the IMP1 synchronous RNA profile (compare with Storms *et al.*, 1985), while the top arrow indicates the expected location of the 3.3 Kb IMP1'-'lacZ band, based on the relative mobility of the large rRNA band. Note that the faint 3.3 Kb band observed at the expected location is only apparent at $t=60$ and $t=70$, although densitometric scanning also detected bands of the same mobility at $t=50$ and $t=80$. The low abundance of the 3.3 Kb species presumably reflects the numbers of transcripts from the single copy, chromosomally integrated pRS306, as opposed to high RNA levels from the multi-copy pRS535 and pRS269 plasmids used for Figures 34 and 36. The identity of the faint, higher MW bands is unknown. The "shadow" at the bottom of lanes 1-4 is due to non-specific annealing of probe. All lanes were loaded with 15 μ g of total RNA.



1 2 3 4 5 6 7 8 9

blotting errors.)

When pRS269-AH22 was monitored during synchronous growth, its IMP1'-lacZ RNA, probed with IMP1 (HindIII - BamHI) DNA, showed a pattern essentially the same as seen for pRS535, as seen in Figure 36A (arrow). With the same blot overexposed, (Figure 36B), the probe detected the lower MW (1.1 Kb) IMP1 RNA (arrow) showing typical periodic behavior, including the characteristic peak at 20 minutes. Note that the weaker signals from lane 3 (t=0) and from lane 11 (t=80) were due to loading errors.

Because the unexpected fusion RNA profiles might have been caused by the presence of the fusion genes on a multicopy plasmid, the third synchrony experiment shown in Figure 37 was designed to detect RNA from a single copy fusion gene, in a pRS269 derivative, pRS306, integrated at leu2. It should be noted that pRS306, like pRS665, is deleted for 2μ-circle DNA and for the HindIII to PstI 5' region of IMP1 DNA (Figure 5A). Since the loss of the HindIII to PstI 5' IMP1 region from autonomously replicating plasmids had no effect on Bgl activity (eg. from pRS669-AH22, Table 4), it was expected that fusion RNA levels would be roughly equivalent to IMP1 RNA levels. In addition, the roughly 400 bp of IMP1-derived RNA from the pRS306 (pRS269-like) plasmid, was expected to produce a signal roughly equal in intensity to that of IMP1 RNA, when hybridized to a HindIII - BamHI IMP1 probe.

As can be seen in Figure 37 (bottom arrow), the periodic IMP1 RNA profile (Figure 37, bottom arrow) detected with a IMP1 (HindIII - BamHI) probe is again clearly normal (cf. Storms et al., 1985). In contrast, no IMP1'-lacZ RNA of the expected 3.3-Kb MW (Figure 37, top arrow, based on rRNA mobility) was detected, until a faint band appeared in the second synchronous generation. In terms of a failure to express

fusion RNA at high levels during the first synchronous generation, this result apparently repeats those shown in Figures 34 and 36, and makes it unlikely that the IMP1'-lacZ RNA profile observed in the first two cases was due to the location of the fusion gene (on a replicating plasmid). In addition, the relative level of any potential IMP1'-lacZ RNA from the integrated fusion gene detected with the IMP1 probe (Figure 37) was obviously much lower than the IMP1 RNA levels, although this was to some degree anticipated (see Discussion).

If the profiles observed in Figures 34 and 36 were steplike, then it was possible that the pRS535 and pRS269 RNA profiles could be caused by enhanced stability of the fusion RNA, as was the case for β gal activity in pRS535-AH22. The relative levels of these transcripts during synchronous growth were therefore determined by densitometric scanning of the autoradiograms, then correcting for pBR322 transcript levels in each lane. The plot (Figure 35, top) of the relative IMP1'-lacZ RNA levels from Figure 34B (or 34A) resembled the profile of stable β gal activity monitored during the same experiment (Figure 35, bottom) until cytokinesis at 70 minutes. In contrast, the peak profile of IMP1 RNA (Figure 35, top) from Figure 34A during synchronous growth matched that observed by Storms *et al* (1985), with peak levels at 20 and 80 minutes. Densitometric scanning also suggested a plateau in pRS269-AH22 fusion RNA levels (Figure 36), from $t=20$ to $t=60$ during the cell cycle (not shown). These profiles therefore suggest that much of the 3.3 Kb fusion RNA is relatively stable during the first cell cycle, unlike IMP1 RNA, which may account for the step profile.

C.6.2. RNA from log phase and α -Factor arrested cells

Although the fusion RNA profile could be caused by relative

stabilization of transcript, the reduced levels of first-generation fusion RNA relative to IMP1 RNA could not be explained in this way. I next considered the possibility that the correctly regulated RNA formed a subset of the 3.3 Kb band, causing the true IMP1'-lacZ RNA profile to be masked. It was reasoned that an unregulated RNA transcribed from this region of the plasmid would not respond to α -factor, whereas correctly regulated RNAs should be reduced in the presence of α -factor like IMP1 RNA (Störms et al, 1984; Greenwood et al, 1986). In addition, pRS535, pRS269 and pRS264 encoded IMP1'-lacZ fusion genes would be expected to yield different length RNAs, since pRS269 and pRS264 have almost 300 bp, and 550 bp, more IMP1 DNA, respectively, than pRS535. On the other hand, an unregulated transcript might have a constant size.

To test these two possibilities, RNA was isolated from log phase cultures and from α -factor arrested cultures of some of the lacZ fusion strains studied above. This RNA, isolated from yeast strain AH22 harbouring one of the following plasmids - pYT760-RYP3, pRS306 (a plasmid similar to pRS665 containing the pRS269 BglIII IMP1'-lacZ fusion junction, and integrated at leu2; donated by Lydia Lee), pRS264 (the "pRS264" derivative is deleted for several bp of IMP1 DNA upstream of the pRS264 BamHI IMP1'-lacZ fusion junction [Figure 5] so that the fusion junction is in-frame, M. Greenwood and E. Calmels unpublished; but is still 550 bp longer than pRS535, a difference readily distinguishable on gels), pRS269, pRS535, pRS535-1A or pRS535-10C - was subjected to Northern blotting. The autoradiogram shown in Figure 38 was probed for the presence of lacZ RNA, and for URA3 RNA and the pBR322 transcript.

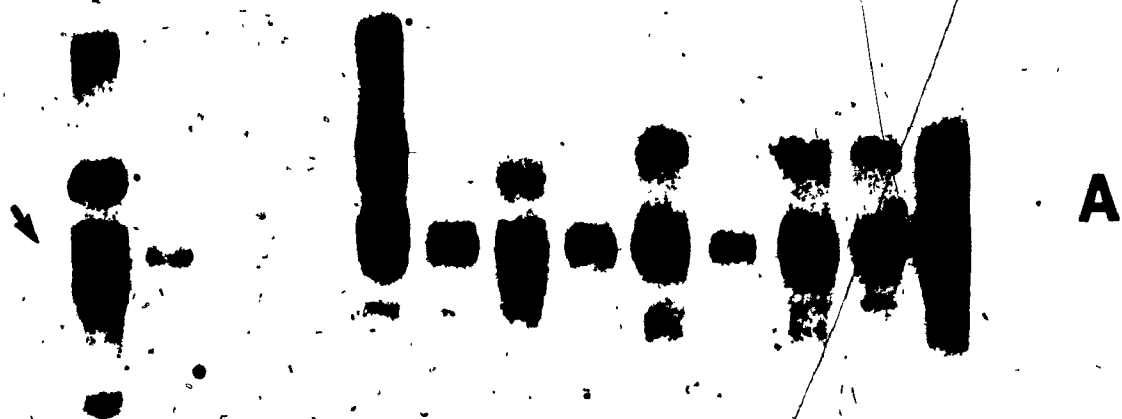
Figure 38A indicates that a common lacZ RNA or group of RNAs of similar size ($3.3 \text{ Kb} \pm 0.4 \text{ Kb}$, arrow), are produced during log phase by these strains, with the exception of the pYT760-RYP3 log phase RNA

Figure 38 A. IMP1'-lacZ RNA expressed from pRS535 and related plasmids in yeast, isolated from log phase or α -arrested cells.

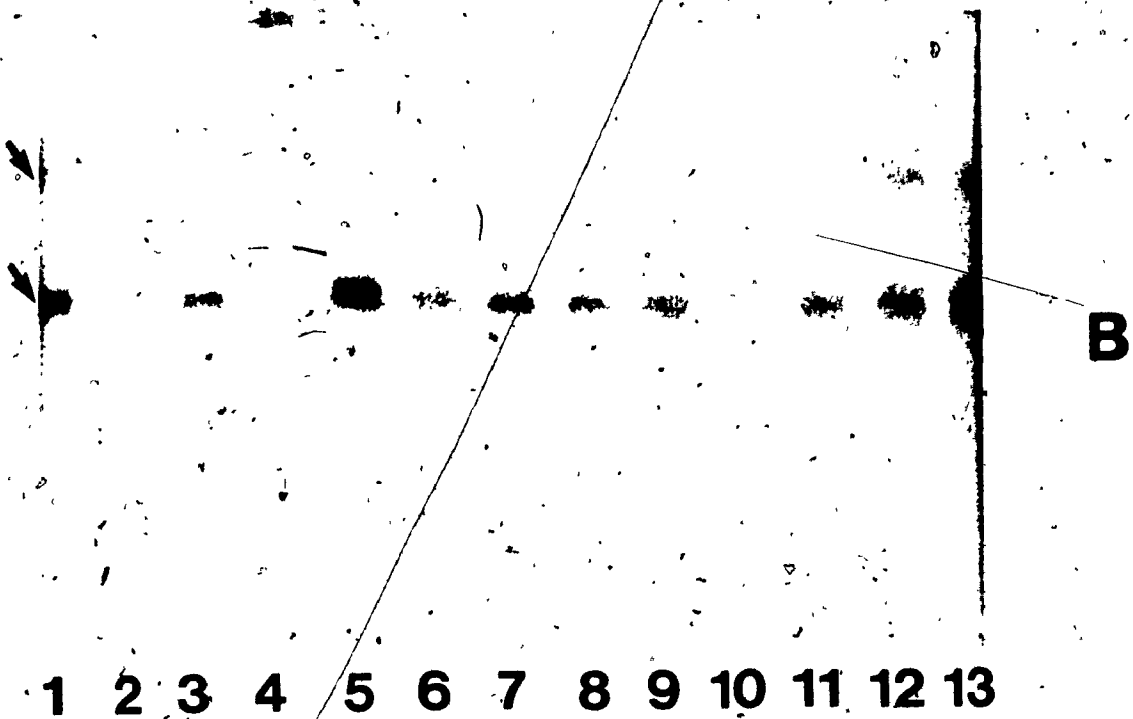
The major transcript size was expected to be greater than or equal to 3 Kb, based on the lacZ coding sequence length (Kalnins *et al.* 1986), and assuming that all -lacZ mRNAs have a common 3' end downstream of the lacZ/lacY boundary. The latter is roughly 50 bp 3' to the lacZ EcoRI site (Figure 6).

Northern blots of IMP1'-lacZ RNA were probed with a BamHI-ClaI lacZ fragment from pMC1871 (specific activity of the probe was $\sim 1 \times 10^7$ dpm/ μ g, exposure time was 4 days). Odd-numbered lanes contain RNAs isolated from log phase cells; even-numbered lanes contain RNA from cells arrested with α -factor. RNAs shown are from pYT760-RYP3 (lanes 1 and 2), pRS306 (lanes 3 and 4), pRS269 (lanes 5 and 6), pRS 2641 (lanes 7 and 8), pRS535 (lanes 9 and 10), pRS535-1A (Δ MluI fragment; lanes 11 and 12) and pRS535-10C (inverted MluI fragment; lane 13). All lanes were loaded with 15 μ g of total RNA. However, two lanes were subject to loading error: lane 2, which was underloaded and lane 5, which was overloaded (see Appendix for sister gel photographs). For quantitation (Table 5), an autoradiogram exposed for a shorter period of time was employed. The arrow indicates the 3.3 Kb fragment, whose average size was estimated from the almost identically sized 3.4 Kb rRNA band detected from sister gels run concomitantly in the same electrophoresis tank (Appendix). Note that the pRS535-AH22 3.3 Kb band width spans approximately 0.6 Kb.

Figure 38 B. URA3 RNA (lower arrow) and a transcript from plasmid pBR332 DNA (upper arrow) from the same blot shown in Figure 34 A, detected with labelled YIP5 plasmid which contains both URA3 and pBR322 sequences (specific activity of the probe was $>10^7$ dpm/ μ g, blots were exposed for 6 days). All lanes contain the same samples shown in A. The upper pBR322 band (≈ 3.2 Kb) ran perceptibly below the IMP1'-loc2 band noted in A. Note that the virtual absence of this band from lanes 3 and 4, which contain RNA from a strain bearing the leu2-integrated pRS306, was expected because of the low copy number of the chromosomally integrated plasmid.



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



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

TABLE 5. - Comparison of β -gal activity/cell with levels of lacZ message corrected for pBR322 transcript levels. Values are all relative to levels from pRS535-AH22.

Plasmid in AH22 ^a	<u>lacZ</u> RNA levels* corrected for the pBR322 transcript log		β -gal activity levels* corrected for cell numbers ($U/10^7$ cells) log	
	phase	α - arrested	phase	α - arrested
pRS535	1.0	0.5	1.0	0.6
pYT76-RYP3	0.7	0.8	1.0	1.7
pRS269	0.5	0.3	2.2 ^b	1.6 ^b
pRS264i	0.4	0.3	≈ 4 ^b	≈ 3 ^b
pRS535-1A	1.5	0.5	0.12	0.17
pRS535-10C	1.7	ND	2.4	1.7
pRS306	0.1	< 0.01	≤ 0.1 ^b	ND

* see Figure 37 for RNA profiles. See Table 4B for log phase β -gal activities. Cultures were pregrown to a density of $\approx 3 \times 10^6$ /ml in selective medium, arrested by the addition of an equal volume of α -factor from an S288C culture, for 80 - 100 minutes.

^b averaged from the three pRS269-type strains shown in Table 4.

^b data from separate experiments (β gal values are from Greenwood, 1986).

(lane 1). The size of the 3.3 Kb transcript was based on mobility of the large rRNA standard.

The 3.3 Kb RNA was reduced in the α -factor-arrested samples relative to log phase samples from pRS269 (Figure 38A, lane 6 vs. lane 5), pRS264i (lane 8 vs. lane 7) and pRS535 (lane 10 vs. lane 9), as would be expected if transcription were reduced during α -factor arrest in these strains. The reductions are quantitated in Table 5, corrected for the pBR322 transcript levels. Furthermore, RNA from the pRS535-10C derivative which contains the inverted MluI sequence (Figure 38A, lane 13, see also Figure 39, lane 9) was somewhat elevated relative to pRS535 RNA (Table 5), and reduced in the presence of α -factor, in agreement with the *Bgl* results from this strain (Table 4). Log phase RNA levels from the pIL35-AH22 strain tested in the same experiment (not shown) also yielded high lacZ RNA levels, again in agreement with the *Bgl* data given in Table 4.

However, the data shown in Figure 38A did not resolve the RNA species expected from pRS535, pRS269 and pRS264i, given the predicted sizes of fusion RNAs (roughly 3 Kb, 3.3 Kb and 3.5 Kb respectively). These estimates are based on the predicted IMP-1 mRNA 5' end location at -60 (Taylor, 1986), and on the published 2.9 Kb length of the lacZ coding region.

In addition, the same 3.3 Kb RNA species, at approximately the same intensity relative to the pBR322 control RNA, are observed from the pRS535-1A derivative lacking the MluI sequence (Figure 38A, lanes 11 and 12). This result is in conflict with the five-fold reduction in *Bgl* accumulation seen in cells bearing this derivative.

Third, the 3.3 Kb band from pRS535-1A is reduced in the presence of α -factor (when corrected for pBR322 RNA; Table 5), whereas *Bgl*

results indicate that this derivative is not regulated by α -factor.

The latter observations are again consistent with the possibility that the correctly regulated fusion transcripts exist as a subset of total RNA homologous to the fusion gene, although the consistently reduced RNA levels in the presence of α -factor are difficult to reconcile. In addition, because of the relative thickness of the fusion "3.3" Kb bands, the failure to detect size differences in RNAs from different sized fusion genes did not eliminate the possibility that a subset of correctly regulated RNAs existed within the 3.3 Kb RNAs.

[The behavior of two other plasmids included in the experiment was as expected. The main RNA band seen from α -factor-arrested pYT760-RYP3-AH22 (Figure 38A, lane 2) is not reduced when corrected for loading errors by comparison with pBR322 RNA levels (Figure 38B, lane 2; Table 5). This result was expected, since *lgal* activity expressed from pYT760-RYP3 was not regulated by α -factor (Figure 30). Furthermore, as was seen in Figure 37, levels of RNA from the pRS306 plasmid integrated at LEU2 (Figure 38A, lanes 3 and 4) were almost undetectable, likely due to the single copy per cell of the integrated fusion gene. In confirmation of this, the pBR322 transcript (top arrow) from the integrated plasmid is also undetectable, while the genomic URA3 RNA band (bottom arrow) is clearly evident.]

In an attempt to locate appropriately-sized transcripts within the rather wide 3.3 Kb band of fusion RNA (Figure 38A), pRS264i, pRS269, pRS535, pRS535-1A and pRS535-10C RNAs (from the same preps stored for eight weeks at -80°C) were electrophoresed more slowly and for a longer distance. Although no internal control was included in this experiment, the amounts of total RNA loaded were very similar based on rRNA band intensities (see Appendix), with the exception of log phase

Figure 39. IMP1'-lacZ RNA expressed from pRS535 and related plasmids in yeast, isolated from log phase or α -arrested cells and fractionated on a 1.5% agarose gel. Northern blots similar to those seen in Figure 38 were probed with the BamHI-ClaI lacZ fragment from pMC1871 (specific activity of the probe was $> 1 \times 10^7$ dpm/ μ g, exposure time was 3 days). Odd-numbered lanes contain RNAs isolated from log phase cells; even-numbered lanes contain RNA from cells arrested with α -factor. RNAs shown are from pRS2641 (lanes 1 and 2), pRS269 (lanes 3 and 4), pRS535 (lanes 5 and 6), pRS535-1A (Δ MIuI fragment; lanes 7 and 8) and pRS535-10C (inverted MIuI fragment; lanes 9 and 10). All lanes were loaded with 16 μ g of total RNA. Note that lanes 2 and 3 were overloaded (see Appendix for sister gel photographs). The arrow indicates the 3.3 Kb fragment, whose size was estimated from the almost identically sized 3.4 Kb rRNA band detected from sister gels run concomitantly in the same electrophoresis tank (Appendix). The RNAs shown in Figure 39 were from the same RNA preparations as shown in Figure 38, rerun after two months storage at -80°C in the DMSO/phosphate pre-denaturation buffer (Thomas, 1982). Note the "processed" appearance of the major band(s) at 3.3Kb (see the actin band(s) in Figure 34C).



pRS269-AH22 RNA (lane 3), which was apparently overloaded. Interestingly, two "3.3 Kb" RNA species were resolved (Figure 39, arrow). One species appears to have a constant size in all the plasmids. The second species appears to vary in size between pRS264, pRS269 and pRS535. For example, the lower, more diffuse "3.3 Kb" bands observed from pRS535 and its derivatives (lanes 5-10) are shorter than both the pRS264i bands (lane 1) by approximately 0.5 Kb, as predicted.

Therefore the possibility that the correctly regulated fusion RNA exists as a subspecies of the 3.3 Kb RNA cannot be eliminated. However, Figure 39 tends to confirm the other conflicting data shown in Figure 38. That is, the absence of the MluI fragment does not appear to affect total fusion RNA levels, although its orientation may have an effect. The alternative possibility, that the transcript levels are in fact unregulated, is considered in the discussion. A third experiment (not shown) using different isolates from the same strains gave similar results.

Table 5 compares the relative amounts of total lacZ RNA from the different fusions shown in Figure 38 with their relative β gal activities (all relative to pRS535-AH22; the β gal activities shown are from two separate experiments; results were quantitated by densitometric scanning across the 3.3 Kb band). As shown in Table 5, none of the log phase RNA values deviate from the pRS535 standard by more than a factor of 2.5 (with the exception of the pRS306-AH22 RNA), whereas the log phase β gal activities of the different strains vary considerably.

Northern analyses could therefore not confirm that fusion mRNA levels fluctuate in a cell cycle dependent fashion, as predicted from the behavior of the translated fusion proteins, although the contradictions could be explained, in part, by the presence of additional transcripts from this region.

These results were not totally unexpected. In spite of the consistently demonstrated value of lacZ fusions in studies of regulated gene expression in yeast (see Introduction), Northern analyses of RNAs from lacZ fusions in yeast have rarely been published, and then only from fusion genes integrated into chromosomal DNA (cf. Hinnenbusch, 1986). The unpublished observations of several groups (XIII International Symposium of Yeast Biologists, 1986) indicate that, while E. coli lacZ mRNAs are transcribed and translated in yeast using the yeast regulatory signals (see Introduction), the overall efficiency of these processes is likely to be reduced. Work has therefore been initiated by others in our lab to detect a correctly regulated subset of fusion RNA using primer extension (E. Calmels), and to confirm the phenotypes predicted by the lacZ fusions for native IMP1 RNA (E. McIntosh).

To summarize the results, the levels of translatable IMP1'-lacZ mRNA from pRS535, as detected by Bgal activity, appear to be regulated in cis by an element located 5' to the structural gene, and 5' to several predicted transcriptional start sites. This element, at least partially contained on the 37 bp MluI fragment (Figure 3), appears to be required for three functions: relatively high levels of translatable RNA, periodic fluctuations in translatable RNA, and decreases in translatable RNA levels caused by α -factor. These results therefore supported the hypothesis, made in the Introduction, that the MluI fragment constituted a 5' CCR element. Because of the upstream location of this element, the "Mlu" element tested was presumed to be involved in transcriptional regulation. This could not be confirmed, however, since preliminary Northern analyses of fusion-encoded RNAs were unable to resolve the predicted regulated mRNA species. Finally, the data support the hypothesis that TS is regulated post-translationally, since the Bgal

TS'-Bga1 protein fusion expressed from pRS269 is periodically unstable.

D. DISCUSSION

This study reports certain aspects of the regulation of the yeast IMP1 gene which encodes thymidylate synthase (TS). Using synchronous populations of S. cerevisiae cells generated by the α -factor arrest-release method it was shown that active TS accumulates near the beginning of S phase (Figure 1). These results are in basic agreement with the data obtained from several higher eucaryotic organisms, showing that the timing of expression of TS activity is cell cycle dependent, with maximum activity occurring during S-phase (Bachmann et al., 1983; Navalgund et al., 1980; Rode et al., 1980).

The periodic increase in TS levels is determined primarily at the level of its transcript (Figure 2). Elevated transcript levels are restricted to a portion of the cell cycle following START (as defined in the Introduction) and peaking near the beginning of S period.

It had previously been shown that yeast histone expression is also cell cycle regulated at the transcript level. Subsequent to the results showing that TS mRNA was periodically expressed, the yeast HQ, cdc8 and cdc9 genes were likewise reported to be CCR genes (see Introduction for references). Interestingly, all of these genes are involved in chromatin metabolism. These results suggest that, unlike the majority of yeast proteins studied to date (Elliot and McLaughlin, 1978), a subset of proteins which are involved in chromosome metabolism accumulate periodically.

Once TS accumulation was shown to occur in a cell cycle dependent manner, which was primarily determined at the level of its transcript, I then began to study the mechanisms regulating its accumulation. Part of

this study involved deletion analysis of the IMP1 regulatory region. Because TS is an essential enzyme required for the growth of wild type yeast, introducing mutations at IMP1 might have induced undesirable pleiotropic effects on the cell. To minimize such effects, I chose to study IMP1 regulation using an approach which did not involve the generation of mutations at the chromosomal IMP1 gene. The method I chose to utilize involved placing the β -galactosidase - encoding E. coli lacZ gene under the transcriptional and translational control of the 5' regulatory region from IMP1. It was reasoned that the expression of this hybrid, non-essential gene in yeast would ~~direct~~ expression of the chromosomal IMP1 gene.

I therefore examined the expression of a TS'- β gal protein fusion, expressed from autonomously replicating IMP1'-lacZ fusion plasmids. The periodic accumulation of β gal activity was confirmed to be determined at both the level of the transcript, and post-translationally, as is the case for TS regulation. This was accomplished by monitoring β gal activity in yeast cells bearing two different plasmids, pRS535 and pRS269.

β gal activity expressed from the plasmid pRS269 was shown to be unstable, in a manner which resembled TS itself. In particular, the peak in activity of these enzymes during S phase shows that they are both unstable. The pRS269 plasmid contains approximately 1/3 of the IMP1 coding region (Figures 5a and b). This region is evidently translated into a polypeptide which, when fused to other polypeptides (β gal, or, in a normal situation, to TS), confers instability on the product (compare Figures 1 and 23).

In contrast, the activity of the shorter protein fusion product from

pRS535 was stable under all conditions tested, and accumulates in a stepwise fashion during the cell cycle. The periodic accumulation of β gal activity from pRS535 during the cell cycle is therefore not determined post-translationally, but at the level of the transcript. — In addition, the pRS535 profile is consistent with the timing of periodic fluctuations in RNA levels from the IMP1 gene itself (see Figure 18), and so supports the hypothesis that the periodic appearance of TS is regulated at the level of its mRNA. Subsequent deletion analyses of pRS535 derivatives indicated that a 300 bp regulatory region located 5' to IMP1 coding DNA directs cell cycle dependent changes in the rate of accumulation of pRS535-encoded β gal activity. Rates of β gal accumulation are reduced in G1 prior to START and following S phase, as predicted based on the periodic appearance of TS mRNA (Figure 2).

D.1. Preliminary Mapping of a IMP1 CCR element.

The constant stability and cell cycle regulated accumulation of the pRS535 fusion gene product in yeast was exploited to map sequences within the IMP1 5' regulatory region which were responsible for the regulation observed. Specifically, derivatives of pRS535 bearing mutations introduced into the 5' IMP1 regulatory region were transformed into yeast, where their effects on β -galactosidase expression were tested. Several of these mutations deleted the 5' IMP1 MluI sequence (Figure 1.3, Figure 3), whose association with cell cycle dependent regulation was proposed in the Introduction. In support of this hypothesis, cell cycle stage dependent regulation of β gal activity accumulation was lost in the MluI-deleted derivatives.

The results of the deletion analyses are summarized below. Thick lines

indicate TMP1 DNA remaining.

<u>Construction</u>	<u>Plasmid in yeast</u>	<u>Bgal Act.</u>	<u>Periodic Expression</u>
<u>HindIII</u> _____ ATG	pRS535	100%	+
_____ <u>PstI</u> _____ ATG	pRS669	100%	+
_____ <u>5'MluI</u> _____ ATG	pTL35	300%	+
_____ <u>3'MluI</u> _____ ATG	pTL31	4%	-
_____ <u>ΔMlu</u> _____ ATG	pRS535-1A (pTL32)	15%	-
_____ <u>inverted Mlu</u> _____ ATG	pRS535-10C	250%	+

The minimum sequence of DNA sufficient to evoke a pRS535-like periodic step pattern of Bgal activity from TMP1'-lacZ fusions in synchronized yeast cells was a 310 bp sequence of 5' TMP1 DNA bordered by the PstI and Sau3A sites shown in Figure 3. When this sequence is present in pRS669, a periodic increase of Bgal activity occurs (Figure 24). The periodic accumulation of Bgal activity from pRS535-AH22 or pRS669-AH22 corresponds almost exactly to the periodic appearance of new buds in the culture (Figure 41). The timing of the latter is an accurate estimator of the onset of DNA synthesis in AH22 (Figure 1).

Furthermore, the 160 bp immediately 5' to the translational start site, up to and including the MluI fragment, may be sufficient for regulating periodic expression. This observation is based on the periodic β gal activity profile produced from the pTL35 fusion plasmid, (Figure 28) which contains only these 160 5' bps of IMP1. These results show that the MluI fragment is required for periodic expression and suggest that the element positively affects the level of expression.

The pTL31 transformant's accumulation of β gal activity was too low to determine an accumulation profile in synchronous cells. However, based on its failure to respond to α -factor (Figure 33), residual expression from pTL31 is predicted not to be cell cycle regulated.

Finally, the accumulation of β gal activity in a periodic fashion (Figures 21 and 22) is highly correlated with the START-dependent response to α -factor (Figure 30). Loss of the MluI fragment seems to remove both forms of regulation (pRS535-1A-AH22; Figures 25-27 and Figure 30), but the suggestion that these are synonymous cannot be supported at this time, since the pLG667-Z control strain (Figure 8) appears to show START-dependent regulation of β gal activity (Figure 20), yet its RNA levels are reported not to fluctuate periodically (Osley *et al.*, 1986). Therefore, as proposed in the Introduction, it may be that all CCR genes are START-regulated, but not all START-regulated genes are periodically expressed.

In drawing these conclusions I note that the profiles observed were subject to the inevitable variability of physiological measurements, and that the synchrony profiles were interpreted, not best-fits. However, the differences in β gal activity profiles from the relevant fusions were considered sufficient to justify the interpretations.

Figure 40. Comparison of relative increases in β gal activity/ml during the cell cycle, between the AH22 derivatives containing pRS535 with an intact IMP1 5' regulatory region, or pRS535-1A with the IMP1 5' MP1 fragment deleted, or pRS535-10C with the same fragment inverted. Note in particular the lack of down regulation from pRS535-1A.

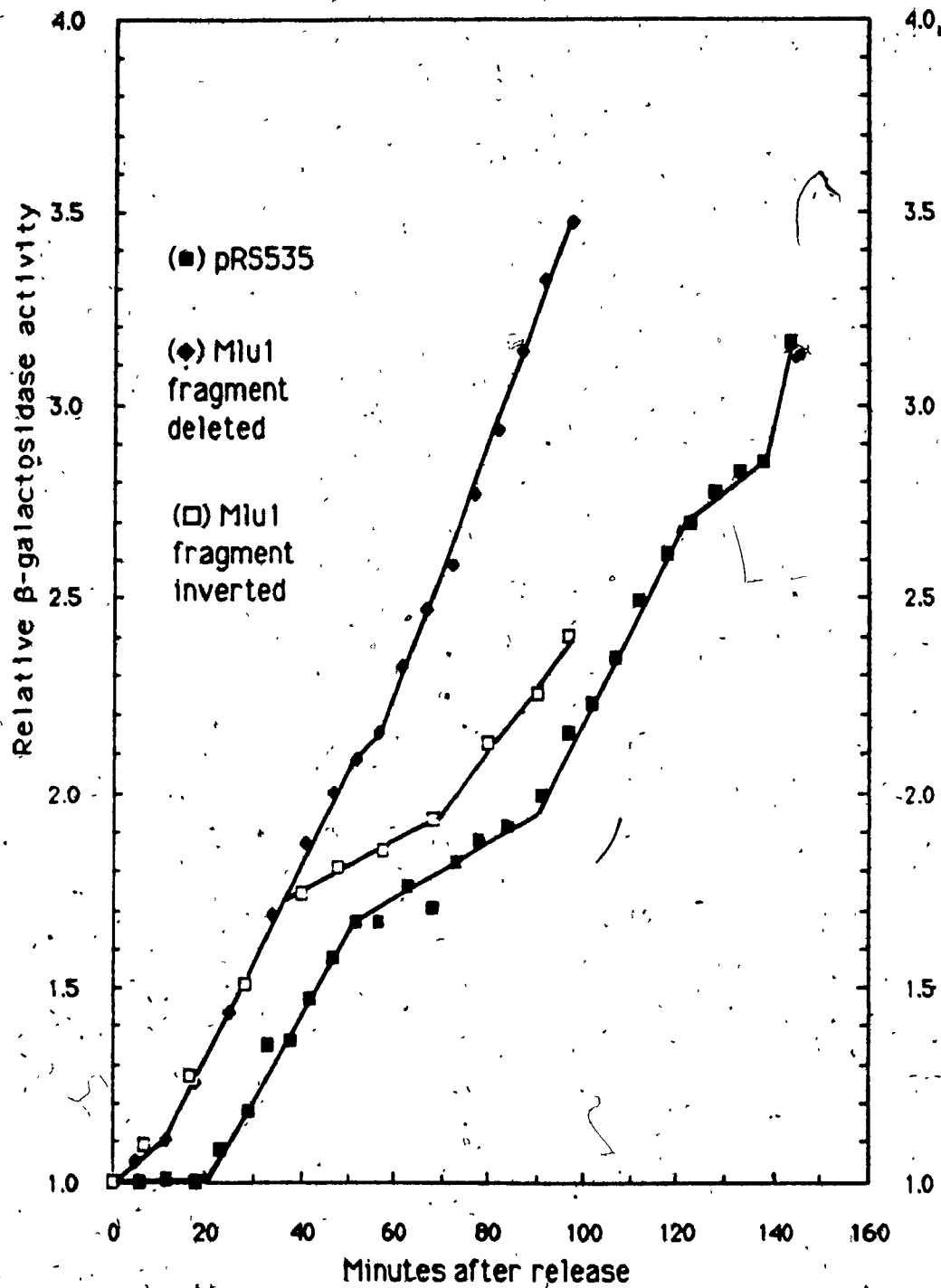
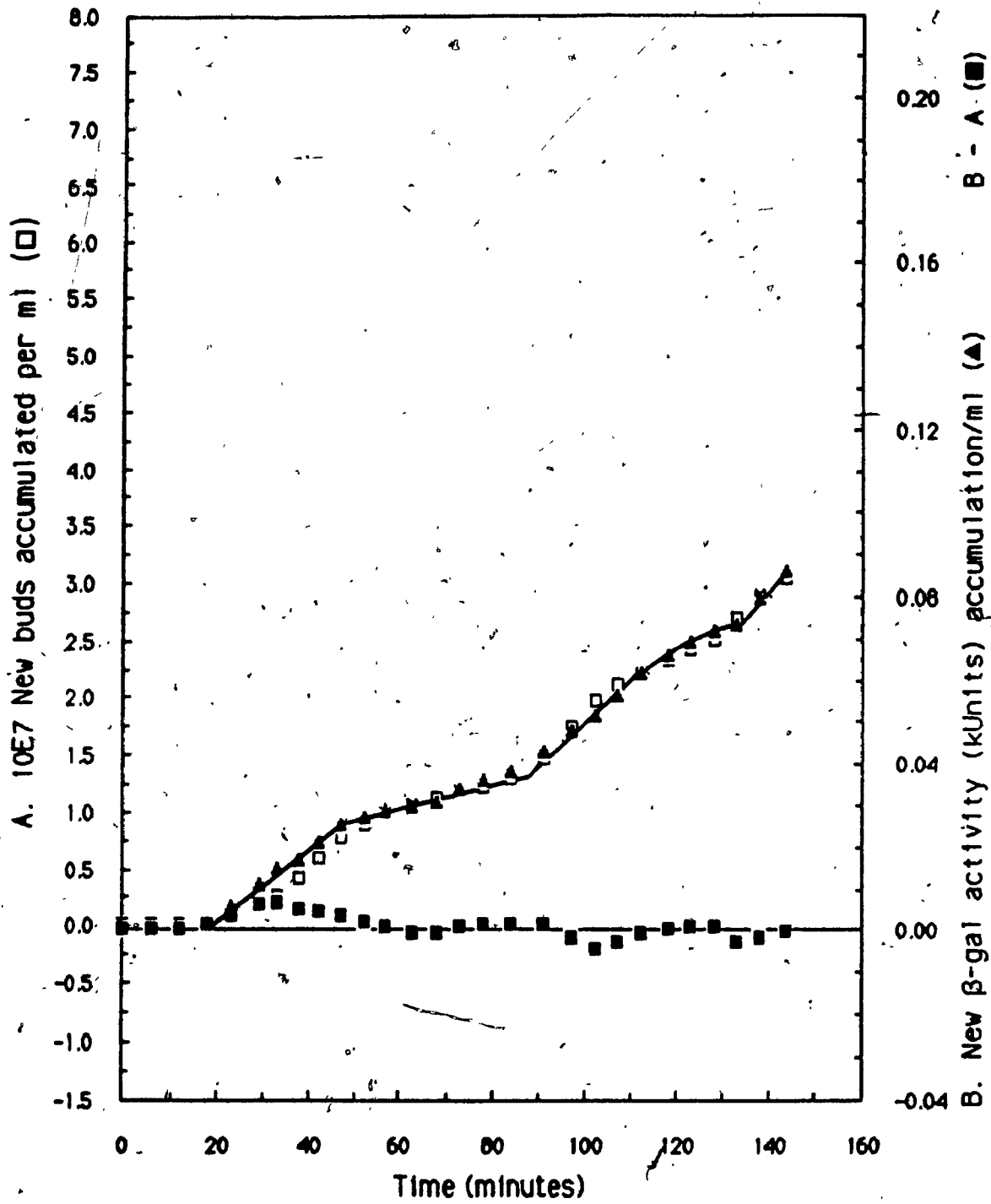


Figure 41. Correspondence of budding activity with Bgal activity in synchronized pRS535-AH22 cells (data from Figure 21) during the cell cycle. Bgal activity is in kUnits (nmol/min.ml). The differences between the two step curves has also been plotted (B-A), after setting the relative levels of both step curves to 1.00 at the t=18 minute point.



For example, Figure 40 shows a replot of the β gal activities from Figures 21, 26 and 29 representing β gal activities from pRS535, from the Δ Mlu derivative pRS535-1A, and from the pRS535-10C derivative, respectively. Here the loss of the step-profile from the Δ Mlu derivative is quite apparent. The pRS535-10C construction contains the MluI fragment inserted at the correct site in inverted orientation (Taylor, pers. comm.). Its behavior demonstrates that the MluI fragment, although required for periodic expression of β -galactosidase, can function in an orientation-independent fashion.

The MluI region appears to be required for a cell-cycle stage dependent increase in the accumulation of the pRS535 fusion gene product, between START and the end of S phase. Since loss of the element reduced rates of β gal activity accumulation, the element appears to have a positive role.

Evidence that the MluI region alone is sufficient to cause a step-like increase in β gal activity awaits its introduction in front of a non-cell cycle regulated gene, such as the RYP3'-lacZ gene fusion (Figures 8 and 19), or the pLG669-2, CYC1'-lacZ fusion. (Figures 8 and 20). The CYC1'- promoter also bears one MluI sequence, just downstream of UAS2 ("upstream activating sequence") at position -163 (Guarente et al, 1984; see below). This allows easy insertion of the IMP1 MluI fragment directly into this plasmid. However, the CYC1 promoter is apparently repressed in α -factor arrested cells (Figure 20 and Appendix), which may complicate a study of the regulatory effects of the MluI fragment in this system. Given the START-dependent regulation of the CYC1'-lacZ fusion gene product, the pre-existing MluI sequence at this locus may further implicate Mlu sequences as 5' regulatory elements, but may also

complicate the interpretation of gene expression results using derivatives of this plasmid.

In contrast, the pYT760-RYP3 plasmid yields a "continuous linear" activity profile during the cell cycle. This lack of induction would simplify analyses of altered activity profiles from this plasmid. A linear profile of enzyme activity through a synchronous cell cycle is rare in S. cerevisiae (although quite common in S. pombe, Mitcheson, 1971). Insertion of sequences 5' to lacZ are less simple for this plasmid, but there are PstI and ClaI sites in the proximity of the promoter sequence (Figure 8). A further advantage to using this plasmid to study the MluI sequence is that its Bgl activity (Figure 19) and its RNA (the major band, after correction; Table 5) both appear to be unaffected by α -factor treatment.

D.2. MluI elements are found 5' to other CCR genes expressed early in the cell cycle.

As noted in the Introduction, GGCG repeats like the MluI element are implicated in the regulation of periodically expressed genes from several different organisms.

First, 5' MluI sites forming part of a GGCG dyad, are found within the first 200 bp upstream of the periodically expressed CDC9, CDC8, and IMP1 yeast genes, whose RNA is subject to cell cycle regulation (Figure 42; reviewed in the Introduction), and TOP2 (encoding a DNA topoisomerase; reviewed by Wang, 1985). These genes' 5' repeat element also resembles the CCR region found between the histone H2 genes (Figure 42; Osley and Hereford, 1986). This is at first sight contradictory, since White et al (1986) show different timing of histone

Figure 42. Symmetric 5' regions common to CCR (cell cycle regulated) genes. The genes indicated have all been shown to express periodically regulated RNA, with the exception of IOP2 (see Introduction for sources).

- A. Yeast CCR genes containing the "Mlu" motif (underlined). A consensus sequence, CGCGTTAA is generally separated from a similar CGCG repeat by an "A" - or purine- rich region. A second sequence GAAAAA, is often found between the Mlu repeats, or nearby. (* The sequence numbering was taken from Genebank, while ** the exact IRT1 intergenic region was not available).
- B. Regions resembling the yeast "Mlu" consensus sequence CGCGTTAA, and sequence organization, 5' to human adenovirus genes (see Introduction).

A. YEAST (positions are relative to the translational start codon).**IMP1**

MluI
-150
MluI
-100
 AAT ATTTGGTGAC GCGTTAAATA GAAAAAATGA AAAAGACCTT AATTGACCG TTTCTGAAA TATTTACT ATAGCATAGC

CDC9

-150
MluI
-100
 CCTCCACTTG AGATGGCTGA TGAATTTTT CACCTTAACG CGAAAACCGG TGAAAGTGAA ATTTAGCTGC AATAAGCCAT

(-110 TO -160 inverted- GCAGCTAAATTCACCGGTTTCGCGTTAAAGGTGA AAAAATTCCATCAGCCA)

CDC8

-150
MluI
-100
 GTTGAAAAAT AGCTTAGAAA TGAAGAAGAG AATAACCGCT TCTATCGCGT TGTGACTTTT GACGCGTTAG GCGTGGTGCT
-70
 AAAGATGCGG AAAAAATGA ACGCTTCCC

TOP2

-200
MluI
-150
 CGAATTCCTT ACGAGGCGAT GAGTGAAAGA CGCGCAGTAG GACGCGTCTT TTTTITTTCA GTATAATGTA AACATATTAT
-90 (inverted GAAAAAAAAA)
 TGAATTGCGGAA

HISTONE H3 (copy 2; litrogenic region)

1000*

TAGGACGCCC TTAGGCGTGC CAATAGTTT ACGCGCTTAA TGCGAAGTGC**HISTONE H2A DYAD (at -400)****GCGCGTTAACTATGGTAAAGACGCCC**B. ADENOVIRUS** (position relative to cap site)**E2**

-60
-40
-20
+1
 GACGT AGTTTTGCGG CTTAAATTG AGAAAGGCGG CGAAACTAGT CCTTAAGAGT CAGCGGCAG TATTTGCTGA A

E1B

-60
-40
-20
+1
 TGTTT AACTTGCATG GCGTGTTAA TGGGCGGG GCTTAAAGGG TATATAATGC GCCGTGGGCT AATCTTTGGTT A

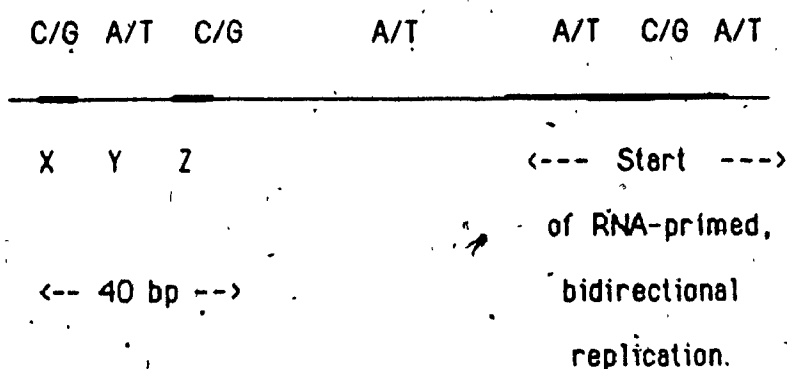
and CDC9 RNA peaks during the cell cycle, which implies that histone genes and the CDC9 gene battery do not share a common regulatory element. However, the histone OCR element in conjunction with the CYC1 UAS causes RNA from a heterologous OCR-CYC1'-lacZ gene to be expressed earlier than histone RNA, in the first cell cycle after α -factor release (Osley and Hereford, 1986). Therefore the later timing of the histone RNA peak relative to mRNA peaks from the other OCR genes tested may be due to factors other than the OCR element, such as transcript stability.

Second, the "5' CGCG-purine rich-CGCG" repeat sequence contained by the two MluI cut sites is structurally similar to repeats found 5' to the periodically expressed adenovirus E1B gene, and E2 gene (cited in the Introduction). This study indirectly supported the hypothesis that the IMP1 5' "Mlu" regulatory element was both homologous and analogous to the mammalian sequences. For example, RNA from the E1B gene, which contains the 5' sequence homology and is regulated by the E1A transforming gene product, is present at the same stage in the cell cycle as IMP1 RNA (eg. at the G1/S boundary). In contrast, a cellular gene, HSP70, whose RNA levels are elevated at a different time in the cell cycle does not contain homologies to the IMP1 MluI element in its 5' region.

Interestingly, removal of only one of the two 5' Mlu repeats from the IMP1 5' regulatory region eliminates cell cycle regulation and reduces expression of the gene product, while 5' deletions removing one of the two adenovirus E2 CGCG-rich sequences also drastically reduces E2 gene expression (Kovesdi et al, 1986).

CGCG repeats similar to the MluI sequence but of unknown function have also been found in yeast mitochondrial (mit) DNA associated with

both replication origins and intergenic spacers (de Zamaroczy and Bernardi, 1986). In particular, the eight ori sequences present in yeast mtDNA contain C/G rich sequences interspersed with A/T-rich regions, as summarized below.



(modified from de Zamaroczy and Bernardi, 1986). Second, the "X-Y-Z" mtDNA consensus ori region is capable of forming a stem loop similar to that formed by IMP1:

IMP1

CAAAC

ATAGAAAAAT

T^TAATATT GG^TGAAGCGTTAA

G

A^TTTATAAA CC^TTTGCGCAGTT

A

GATATC

GT

AATTCCAGAAAA

MitDNA ori "X-Y-Z" sequence

G "X"GGGTCCAATTATTATT T "Y"

CCCAGGGTTAATAATAA C

G "Z"

Could the regulation of a OCR gene such as IMP1 act in cis to influence the onset of chromosomal replication? Although the possibility of OCR elements themselves having ARS activity is very low (eg. Osley *et al.* 1986; this study), the physical association of cell cycle regulated genes such as IRT and HQ with ARS sequences (see Introduction) may not be entirely fortuitous:

Third, the sequence "GGT" precedes the G/C cluster "X" in three inactive mitochondrial ori-like sequences otherwise similar to the above X-Y-Z sequence. The sequence GGT is also found 5' to the first MluI element which resembles the X-Y-Z sequence as noted above. As a result, it is possible that the MluI element constitutes a partial, and inactive, ARS-modulating sequence, albeit with mitDNA homology. Although no evidence for a functional ARS near IMP1 has been noted (unpublished), this may not be the relevant datum. It may be that OCR elements are like the X-Y-Z sequence noted above, required to regulate ARS-like sequences in an as-yet-undetermined fashion, without actually forming the replication origin itself.

Elements like the MluI sequence, dispersed throughout the genome, could therefore have varying degrees of function in regulating either cell cycle related gene expression, or replication origins. Such elements, as pointed out by de Zamaroczy and Bernfield (1986), may have evolved from a common origin, and their duplicated nature suggests that they may have originated as transposable element insertion sites (*ibid*).

D.3. The 400 bp HindIII to Sau3A IMP1 5' fragment contains a novel START-dependent regulatory element.

The MluI 5' regulatory sequences are located upstream of putative

transcription start sites (5' mRNA ends detected by primer extension mapping; McIntosh, pers. comm.) and of DNA "TATA" elements associated with transcription initiation, and so the normal assumption is that the region studied affects transcription. Since the periodic expression of IMP1 as detected from the IMP1-lacZ fusion appeared to be associated with a 5' DNA element, it was of interest to compare its sequence and regulation with other CCR genes. A search of the 400 bp 5' regulatory region present on pRS535 did not identify elements similar to the CACGAAAA sequence of Nasmyth (1985b) nor the related, "16mer" of Osley *et al* (1986) (the search was based on perfect fit of the PuCGAAA "core" sequence of these elements). Since these genes' START dependent regulatory elements can be located more than 400 bp 5' to the translated region (*ibid*), it was possible that the HindIII fragment is lacking this kind of START-dependent transcriptional regulatory element. As argued below, a close examination of the results suggests that this may be the case. However, it is clear that the MluI regulatory region located 5' to IMP1 is necessary for the periodic fluctuations in START-dependent gene expression. The unique nature of this sequence and its essential role in directing periodic expression suggests that it constitutes a novel CCR element, although, as previously noted, the histone CCR may be analogous.

D.4. IMP1 Regulation by the MluI fragment: transcriptional versus translational models.

At first sight, the cell cycle stage-dependent regulation of Bgal accumulation directed by the MluI region conflicts with the preliminary RNA data, if the fusion mRNA is proposed to be periodically transcribed or degraded. These discrepancies were dealt with, to some extent, in the

Results section. However, because the different sized fusion genes did not yield single RNA species of demonstrably different sizes, the lacZ "system", which has told us that the MluI region regulates fusion gene expression in a cell cycle dependent fashion, is obviously not ideally suited for distinguishing whether fusion RNA is transcriptionally or post-transcriptionally regulated by this element.

It remains possible, however, that the correct species exists within the 3.3 Kb band (as was suggested from Figure 39). One suggestion is that the fusion RNA mobility and stability (note that it would have to be both) could be affected by an interaction with the large rRNA species, which migrates close to the 3.3 fusion RNA, at some stage prior to Northern blotting. For example, faint hybridization signals at rRNA bands have been observed for Northern blots probed with genes other than those used here, as well as IMP1 (E. McIntosh, pers. comm.). It is possible that only this interacting transcript is stabilized or transferred, which could account for the lack of a size difference between the different fusion RNAs in Figure 38. Finally, it remains possible that the entire 3.3 Kb species is the correct transcript, and that the lack of a size difference in RNA from the different fusions is due to an artifact of preparation.

Assuming that the 3.3 Kb RNA species is, or contains, the translatable fusion mRNA, this study was unable to demonstrate that IMP1-lacZ RNA was regulated at the level of transcription. It is therefore worth proposing, on formal grounds, an alternative model to that of transcriptional regulation by the MluI element. Interestingly, results cited here which contradict a model of periodic transcription of fusion mRNAs, actually support a model of cell cycle regulation of translation, as

follows.

First, the CYC1-lacZ fusion mRNA is not START regulated (Osley et al, 1986). Yet the same gene fusion directed the START-dependent appearance of a stable Bgal gene product.

Second, a "rebound effect" of accumulation of Bgal activities to pre-arrest levels is observed following α -arrest (Figures 30, 32), as if Bgal activity were "stored" during arrest, in the absence of a similar "rebound" of fusion mRNA (Figures 34 and 36).

Third, the MluI-deleted cells show an 85% reduction in Bgal activity accumulation, yet show no corresponding reduction in fusion RNA levels.

Fourth, the mRNA from α -arrested cells containing the MluI-deleted pRS535 derivative is still START-regulated (reduced by α -arrest), but the gene product from this fusion is not START-regulated, and no longer accumulates in a cell-cycle dependent fashion.

Fifth, START elements required for periodic transcription are not found on the 5' IMP1 regulatory region, as suggested, both by sequence analysis and by the lack of a first-generation peak of fusion RNA which approaches log phase levels. Furthermore, the RNA profile from synchronized pRS535-AH22 resembles that of the HQ gene, deleted for its URS2 START regulatory region (Nasmyth, 1985b).

Sixth, the RNA profile from pRS535 may be due to stabilization of the fusion RNA, which suggests that the half-life of the fusion transcript is increased relative to IMP1 mRNA. But this predicts that levels of the fusion gene product should accumulate rapidly in the second generation; in fact the second generation accumulation of Bgal activity is less than that from the first (Figures 21 and 23).

These results support the interesting possibility that the

translateability of IMP1 RNA is regulated in a cell cycle dependent fashion, but require that this translateability is regulated by an upstream Mlu sequence not predicted to be present on the mRNA. However, the apparent contradiction imposed by the upstream location of the MluI sequence relative to the 5' location of the mRNA ends may be based on our incomplete knowledge of transcription and its initiation in eucaryotes. The most recent evidence in yeast suggests that 5' regulatory elements may, in fact, interact near 5' mRNA ends. For example, the GAL4 UAS is proposed to interact with the GAL1 transcriptional start site several hundred bases downstream, by protein-protein interactions which loop out intervening DNA (Keegan *et al.*, 1986). If this is the case, then 5' regulatory elements could alter the 5' ends of their mRNAs. Alternatively, the assigning of transcription start sites to regions downstream of TATA boxes may be incorrect in certain instances (the potential existence of a primary transcript initiating upstream of HQ was considered in the Introduction).

Therefore the MluI region could either form part of a longer mRNA precursor (long untranslated 5' leader regions are also observed in mammalian TS mRNAs; see Introduction), or influence the downstream IMP1 mRNA in a fashion similar to that proposed for UAS_G regulation. If the MluI region is thus assumed to determine the integrity or location of IMP1 5' mRNA ends, either pre- or post-transcriptionally, an alternative model, the periodic translation of fusion mRNA, can be invoked to account for the RNA results as described above.

This model has two stipulations. First, fusion mRNA must be translated with high efficiency during certain times of the cell cycle, but be translated with very low efficiency at other times. Second,

regulatory sequences required to produce normal levels of TMP1 RNA in a START dependent fashion (ie. produce a larger first-generation peak of RNA) are missing from the gene fusions studies here.

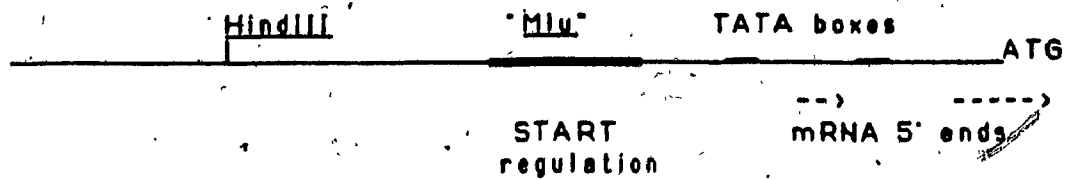
D.4.1. Transcriptional regulation of TMP1.

The two models for TMP1 regulation are summarized in Figure 43. The first model (43A) assumes that the Mlu element is a START-dependent transcriptional enhancer similar to the CACGAAAA sequences reported by Nasmyth (1985a,b) and described in the Introduction. The model presumes that transcriptional regulation accounts for the cell cycle stage-dependent accumulation of Bgal activity observed in this thesis, and does not attempt to reconcile the RNA observations. Confirmation of model A awaits the detection of periodically transcribed TMP1 or fusion mRNA, mediated by the Mlu sequence.

The second model (B; Figure 43) attempts to reconcile the Bgal activity data with the RNA data from the fusions, and so requires some explanation. To begin with, TMP1 is assumed to require a START-dependent URS to generate its periodic RNA profile, which is absent from the TMP1-lacZ fusions studied. The absence of the correct URS from pRS535 could explain the missing first-generation peak of TMP1-lacZ RNA in synchronized cells, just as the START-dependent regulatory element URS2 was required for a first generation peak of HO RNA in α -factor synchronized cells (compare Figure 35 from this thesis with Figure 8 from Nasmyth, 1985b).

The missing TMP1 URS, possibly located in the yeast chromosome XV upstream of the TMP1 sequences studied here, would be responsible for initiating transcription in a START-dependent fashion, similar to URS2 of

A.



B.

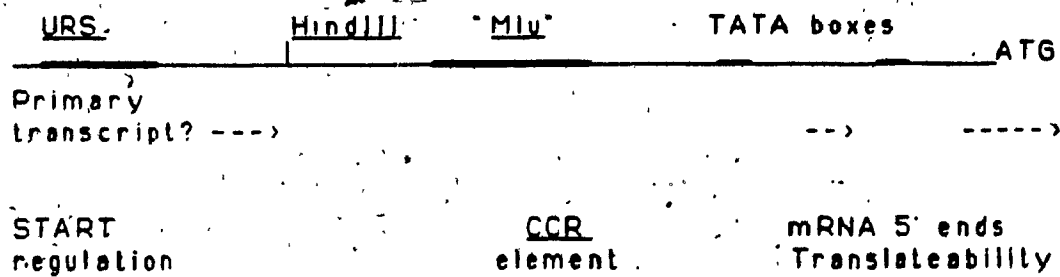
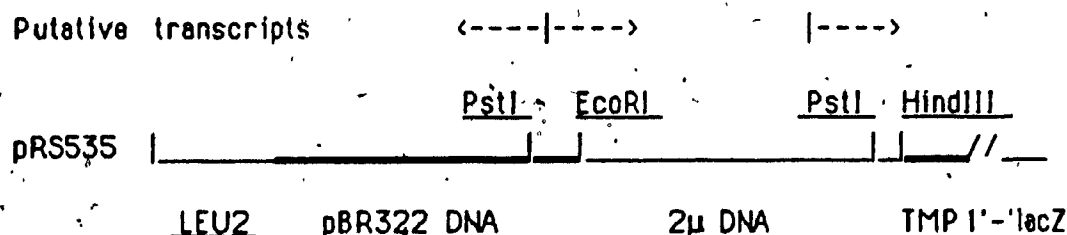


Figure 43. Models of TMP1 regulation. A. A "single element" regulatory model. B. Two-element model.

the HQ gene (ibid, see Introduction).

If a URS located 5' to the HindIII-Sau3A IMP1 regulatory region is required for activating transcription at IMP1, other transcriptional enhancer sequences (UASs) may have been present on pRS535-type plasmids which could substitute for the postulated IMP1 URS to initiate transcription of fusion RNA, although in a non-periodic fashion.

Two potential transcriptional enhancers present in pRS535-type plasmids are shown below.



The first potential "UAS" transcribes the non-periodically expressed "pBR322" transcript(s) (Figures 38B and 34C). This transcript has been suggested to start at a transcriptional activating sequence in the pBR322 amp^r gene promoter near the PstI site (see Figure 6), and to transcribe in yeast a cloned DCD1 gene placed downstream of this region (McIntosh, 1986). Its existence was exploited as a control in these studies (Figure 34C). However, the estimated transcript size is three Kb, too small to enter lacZ DNA in the pRS535-type fusions.

The second potential UAS is in 2μ-circle DNA, near the 2μ-circle PstI site (see also Figure 6). This UAS is predicted to promote a transcript which starts adjacent to and proceeds toward the IMP1 regulatory

region (Broach, 1984). In pRS535, the latter transcriptional enhancer may substitute for the IMP1 START-dependant URS. If this "2 μ UAS"-promoted transcription was less dependent on START than the postulated IMP1 URS, then the essentially non-periodic increase of fusion RNA and the lack of a first generation peak of fusion RNA can be accounted for.

Interestingly, the other potential pBR322 enhancer sequence is positioned within 300 bp of the fusion gene in certain pRS269-derivatives, and can account for the altered β gal activities expressed from these plasmids in yeast. For example, the pRS306 derivative of pRS269 expresses low levels of both β -galactosidase and of lacZ RNA when integrated at the yeast lev2 gene, compared with the levels of IMP1 RNA (Figures 37 and 38). This construction is missing both of the above potential "UAS" sequences. In contrast, a second derivative, similarly integrated, but which contains the pBR322 "UAS" immediately upstream of the IMP1-lacZ fusion gene expresses higher levels of gene product. This tends to support the possibility that expression from the IMP1 5' region can be enhanced by other upstream sequences.

D.4.2. Regulation of IMP1 mRNA from the Mlu region.

As noted at the beginning of Section D., given the absence of its normal URS, translational regulation can account for both RNA and β gal activity expressed from the gene fusions. Assuming it exists, how might the gene accomplish such regulation?

The IMP1 gene could be post-transcriptionally regulated by a 5' leader region, as was proposed for human TS mRNA (Takeishi *et al.*, 1985). One

possibility is that a long unstable precursor RNA which includes the MIU1 region could be periodically processed, under the control of the MIU1 region, to more stable mRNAs of the sizes observed by primer extension (E. McIntosh, pers. comm.). A similar alternative was proposed for HO regulation in the Introduction (section A.2.2). In addition, "processed-transcript" models have been proposed (see Broach, 1984) for the processing of a long 2 μ -plasmid transcript, and for mitRNA by Anderson *et al* (1981; reviewed for yeast mitDNA by Evans, 1983).

Alternatively, it is possible that a subset of the mRNAs produced 3' of the two TATAs (*ibid*) may be translated only after START, under the control of a leader sequence located between the coding sequences and the coding DNA. To account for the observations noted in section D.3.1, this mRNA subset should not be translated in α -factor-arrested pRS535 cells. Upon release of the cells from arrest at START, this subset would be translated with high efficiency, thereby accounting for the "rebound" of Bgal activity following release from α -factor (Figures 30, 32), and thus compensate for low levels of RNA accumulation (Figure 35).

Note that the assumption that a subset of fusion RNA is stabilized (cf. Figure 35) and untranslatable in the fusions; as opposed to the IMP1 gene, is required by both transcriptional and translational models of cell cycle regulation of the gene fusions, in order to explain the unstable behavior of normal IMP1 RNA. However, the translational model could account for why this stable RNA is present, as follows.

In the translational model, the reduction in translation of a CCR subset of mRNAs from either IMP1 or the fusion gene, could coincide with their increased half-life. Stabilization caused by untranslatability has been proposed by others (Hinnenbusch, 1986). For example, most

transcripts from the MluI-deleted pRS535 derivative are presumed to be untranslated, and are accordingly stable (Figure 38). The presence of a similar subclass of transcripts in pRS535 could mask the expression of a CCR subclass of RNAs from this plasmid, without hindering its periodic translateability. As another example, if IMP1 transcription ceases in cells arrested at START, increased mRNA stability could explain the presence of residual IMP1 mRNA observed in cells arrested with α -factor (Calmels, M.Sc. in preparation).

MluI-mediated stabilization could be accomplished here in one of two ways. First, the translateable CCR mRNA could be of a particular size, and changes at the upstream MluI site, could periodically alter the proportion of that size class.

Second, the transcript from a CCR subset of transcripts may receive a START-dependent "tag" depending on the state of the MluI region. The tag could confer either stability, or the ability to be translated during S-phase. For example, the passing of START, or changes at the MluI sequence could alter the ability of the CCR transcript to be capped. (Capping is a ubiquitous event in eucaryotes in which a 7-methyl-GTP is added to the 5' end of an mRNA precursor during its transcription; reviewed by Nevins, 1983).

A proposal of periodic changes in mRNA translateability leads to specific predictions for the result of this interaction. First, IMP1 or IMP1'-lacZ transcripts must be qualitatively altered during the cell cycle (eg. changes in length, secondary structure or base modification). Second, fusion mRNA should also be qualitatively altered, although not necessarily in the same way, in the MluI-deleted strain pRS535-1A. In contrast, a model which proposes that translation is unregulated would

predict only periodic changes in levels of RNA. Such models would include periodic transcription or periodic processing of a longer transcript.

The possibility that a 5' URS is required for START-dependent regulation of IMP1, as was the case for HQ, is currently being tested, by cloning chromosomal sequences located 5' to IMP1, and asking whether START-regulation of the plasmid-encoded fusion genes is altered when these sequences are present next to the pRS535-encoded IMP1-lacZ gene fusion (L. Lee, pers. comm., E. McIntosh, pers. comm.).

D.5. CCR regulation of cell cycle length.

A CCR gene such as IMP1 would be expected to be periodically expressed following START (see Introduction). As such both IMP1 RNA and gene product should be (and are) induced upon release from α -factor or after release from G_0 . This study suggests that the observed periodic expression, of both IMP1 RNA and its TS gene product, is due to a combination of synthesis and a short half-life. In addition, I have introduced the possibility that the intermediate process, translation, may also be START-dependent for CCR genes. Could the timing of any of these START-dependent forms of gene expression be related to a role for CCR genes as cell cycle "trigger" proteins, as proposed in the Introduction? Two apparent contradictions to this proposal actually suggest a simple mechanism by which CCR gene products could trigger START.

The first contradiction is as follows. How can a gene whose expression is not drastically increased prior to START, possibly encode the same protein whose accumulation is responsible for initiating START? Second, Singer and Johnston (1983) demonstrated that START can occur

prior to mitosis in log phase cells which are recovering from cell-cycle restraints, and so cells can initiate DNA synthesis almost immediately on completion of mitosis. How could the cells build up trigger protein in G1 under such circumstances?

Both contradictions can be resolved by considering the fate of unstable trigger proteins synthesized in the previous cell cycle. If the trigger protein were not entirely degraded in rapidly growing cells, prior to the next cell cycle, then the residual levels of the trigger protein, plus new trigger protein synthesized at any stage prior to START, would be what determine the length of time spent in G1 prior to START. This combination must only be sufficiently high to stimulate the rapid synthesis of new trigger protein following START (ie- the trigger protein stimulates its own synthesis after START). In this scenario, the newly synthesized trigger protein is capable of function in the current cell cycle, but could also be a reservoir of trigger protein for the following generation. Therefore cells could continue to cycle and pass START, even if there was no G1, provided that the reservoir of unstable protein was sufficiently high. In slower growing cells which contained a pre-START G1, the passing of START could then be mediated prior to START by the balance of its (slower) synthesis and degradation, in turn mediated by environment.

D.6. Raison d'être for periodic mRNA from CCR genes.

Finally, if the formation of unstable CCR gene products determines START, and if START can precede mitosis, then these products could also be translated before mitosis, from mRNA synthesized in the preceding generation. Sensitive regulation of cell cycle length in rapidly growing

cells, by an unstable protein, may thus require that its mRNA also be cell cycle regulated, so as to not overproduce the critical protein for the following generation, in case of changes in growth conditions. This could be accomplished either by synthesizing highly unstable RNAs such as are seen from CCR genes, or by regulating their translateability as proposed above, or both.

White et al (1986) have questioned the necessity for periodic mRNA expression, based on the observation that mRNA from the Schizosaccharomyces pombe gene for DNA ligase (analogous to the yeast CDC9 gene) is not periodically expressed. For yeast, I have suggested that periodic CCR gene expression may determine the ability to pass START, which is also regulated by START. The above arguments provide a *raison d'être* for cell cycle regulated enzymes and mRNAs from yeast (and mammalian) CCR genes, in addition to their periodic involvement in DNA synthesis. Furthermore, since growth rates in S. pombe appear to be regulated during G2 (Mitcheson, 1971), this organism may be subject to other forms of growth regulation. CCR gene batteries, at least in G1-arrest organisms (*ibid*), could thus supply the theorized unstable proteins whose buildup, regulated primarily at the RNA level, have been proposed to initiate a cell cycle (Donachie, 1968).

D.7. Future Directions.

Just as the relationship between periodic gene expression and CCGG repeats remains unresolved, so does the exact nature of the control exerted by the MluI CCR on gene regulation. Although the CCR functioned effectively to produce a periodically expressed gene product, it may have to be coupled with a particular type of UAS sequence to produce

periodically fluctuating mRNA levels, as was suggested for the isolated histone OCR element and for HQ regulation (see Introduction). Alternatively, the MluI element may function alone as a OCR element, but the IMP1 gene may require, for periodic mRNA fluctuations, additional signals found within IMP1 RNA but not found on the lacZ gene.

Future studies to determine whether the MluI OCR acts pre-transcriptionally, as is predicted from its location, should thus be focused on the metabolism of RNA, from a system containing more 5' and 3' sequences from the IMP1 gene itself, if possible, so that each parameter inferred in this study can be examined separately. A simple insertion of foreign DNA within a IMP1 gene cloned at a second site within the yeast genome would provide a useful model, which would avoid the limitations of the IMP1-lacZ fusions noted here.

Finally, if the MluI sequence is found to modulate RNA transcription (or translateability) from its position 5' to the TATA elements as proposed above, our understanding of the regulation of nuclear eucaryotic transcription may have to be revised. The obvious next steps in this approach will be to examine the 5' mRNA ends of IMP1 and the IMP1-lacZ fusions, first to determine if these ends map to the same sites, second to examine if qualitative changes occur in IMP1 RNA stability or translateability, and third to investigate how the "Mlu" sequence might alter expression of other, non-OCR genes.

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APPENDICES

Figure A.1. Photographs of ethidium bromide-stained sister gels to those shown in Figures 39(A), 36 (B), 34 (C) and 35 (D). Equivalent amounts of RNA were loaded in each lane. From the left, the corresponding lanes in Figure 39 for A. are, respectively, lane 9, two lanes of undenatured RNA, then lanes 1,2,3,4,5,6,7 and 8. For B. the corresponding lanes in Figure 36 are, from the left, lanes 1,2,3,4,5,6,7,8,9,10,11,12 and an undenatured sample. For C. the corresponding lanes in Figure 34 are, from the left, lanes 1,2,3,4, then an undenatured sample of pRS258 (instead of denatured log phase pRS269), then lanes 6,7,8,9, 10,11,12,13 and 14. For D. the corresponding lanes in Figure 35 are, from the left, lanes 1,2,3,4,5,6,7,8,9,10 and an undenatured sample.

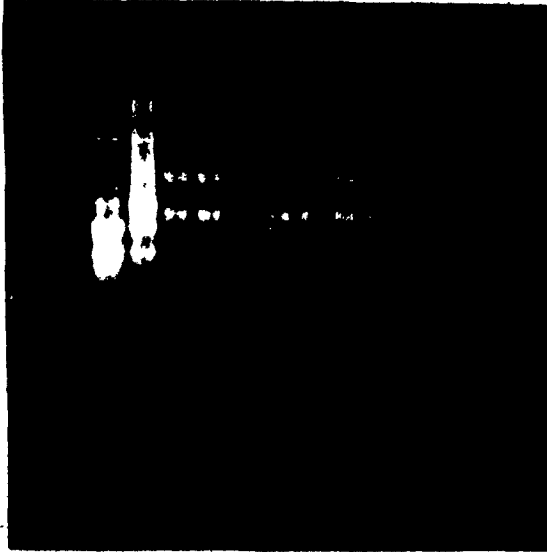
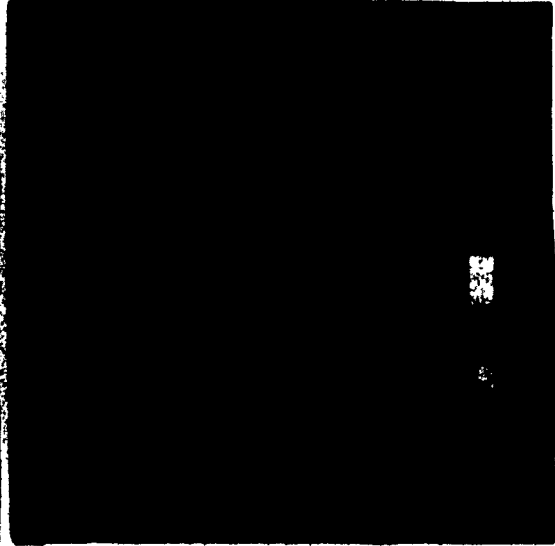
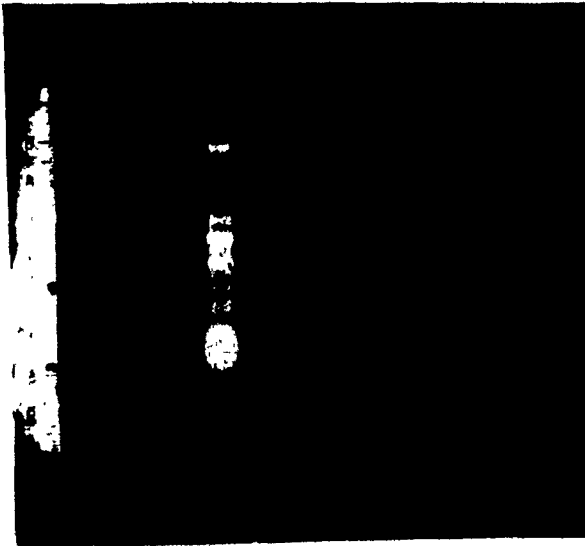
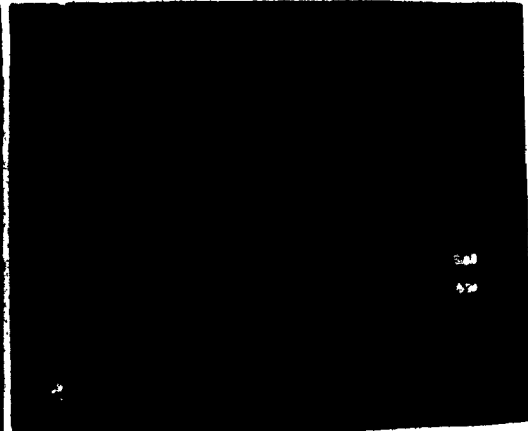
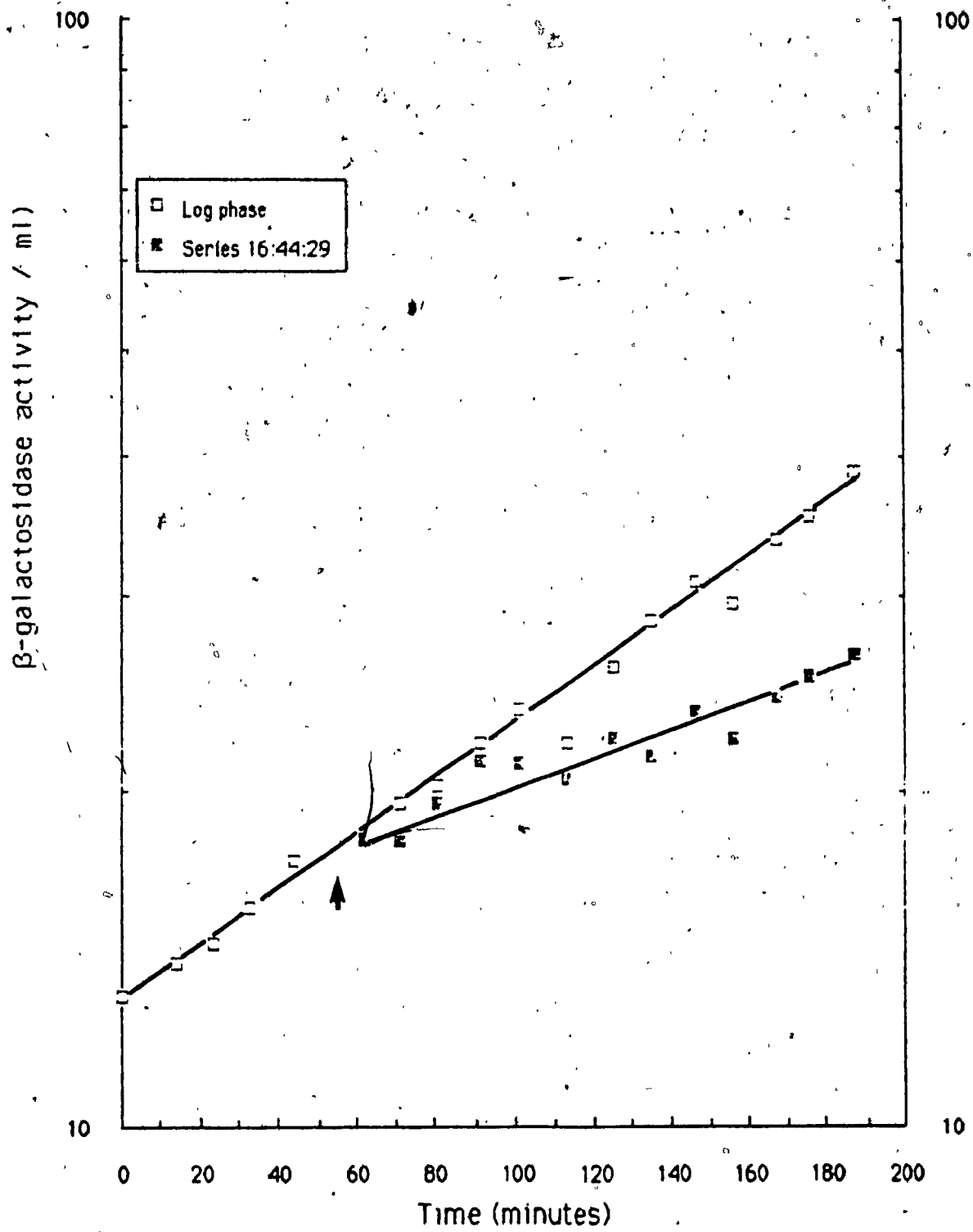
A**B****C****D**

Figure A.2. Immediately divergent β gal activity profile (dark squares, series 16:44:29) during α -arrest, from the AH22 derivative strain RS849 transformed with pRS845 (pLG11), from the β gal profile of the same culture without α -factor (open squares). The arrow indicates the time of α -factor addition. Activity is in nmol/min.ml, and the culture density was $\approx 10^7$ /ml.

The plasmid pRS845 contains the CYC1-lacZ HindIII - SstI fragment from pL0669-Z (pRS769). For this experiment, 100 μ g of α -factor in 100 μ l of water was added to a 100 ml culture (selective media), while a parallel culture was not tampered with. Duplicate samples were assayed for β gal activity at each time point using N2(1)/Brij permeabilization. Duplicate activities per sample did not deviate from the means shown by more than 3%. The timing of the α -arrest was such that the cells were 90% unbudded at the 160 minute sample, but had started budding by 180 minutes.



A. 3. Alternative calculation of β gal Activity vs TS activity levels, per cell.

β gal activity accumulates in pRS535-AH22 log phase cells at about $2500 \text{ U min}^{-1} \text{ mg protein}^{-1}$ or $150 \text{ U min}^{-1} 10^7 \text{ cells}^{-1}$ (Tables 3 and 4). Rotman (1970) estimated that, for the *E. coli* enzyme, there were 2.6×10^9 enzymes per 1000U. Therefore the native β gal enzyme is estimated to account for 0.005% of total yeast protein, and 40 molecules per cell (assuming full activity. The tetrameric, active form of the β gal has a 540,000 MW; reviewed by Zabin and Fowler; 1970). In comparison, estimates of TS per cell indicate that TS is present at 0.005% of total cell protein, (≈ 1000 enzymes per cell; Bisson and Thorner, 1981). Since TS exists as a 70 kD dimer, there is roughly 12.5 times as much active TS produced from the single copy IMP1 as there is active β gal produced from pRS535. If plasmid copy number ($\approx 20/\text{cell}$) is taken into consideration, there may be as much as a 200-fold reduction in enzyme produced per pRS535 fusion promoter. However, this value is a "worst case" scenario, and the higher V_{max} of pure β gal vs the TS'- β gal fusion enzyme may simply reflect cumulative reductions in efficiency of expression at all three levels of catalysis, transcription and translation.