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Transcriptional regulation of the yeast cytochrome *c* gene

(cloned *cycl* gene/RNA half-lives)

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ABSTRACT DNA from the cloned yeast iso-1-cytochrome *c*, *cycl*, gene was used in a hybridization assay to measure levels and rates of synthesis of *cycl* RNA. Derepressed cells synthesized *cycl* RNA at 6 times the rate of that of glucose-repressed cells. Upon glucose addition to a derepressed culture, the transcription of the *cycl* gene was repressed within 2.5 min. The half-life of hybridizable *cycl* RNA was determined to be 12–13.5 min under repressed and derepressed conditions and during repression. The results demonstrate that the expression of the *cycl* gene is subject to transcriptional regulation.

The synthesis of messenger RNA molecules from eukaryotic genes involves not only transcription, but also polyadenylylation of the 3′ end, 5′-capping, and, for many genes, the removal of the intervening sequences from the initial transcripts. These complex processing requirements led to the proposal that eukaryotic gene expression, unlike that in bacteria, is primarily regulated at steps subsequent to transcription (1). To explore this question, we have tested for transcriptional regulation of yeast *cycl* gene expression. The *cycl* gene codes for iso-1-cytochrome *c*, which comprises 95% of the cytochrome *c* in yeast cells (2). Synthesis of iso-1-cytochrome *c* is regulated by catabolite repression; glucose represses the rate of cytochrome *c* protein synthesis and cellular levels of translatable *cycl* RNA (3, 4). Our objective in this study was to determine whether glucose repression acts at the level of *cycl* gene transcription.

Recently, the DNA of the *cycl* gene was isolated by molecular cloning in *Escherichia coli* (5). The availability of *cycl* DNA in quantity makes possible an assay for the rate of *in vivo* *cycl* mRNA transcription by DNA excess hybridization of pulse-labeled RNA. The results obtained with RNA preparations from glucose repressed and derepressed yeast cultures show that *cycl* gene transcription is regulated. The magnitude of this transcriptional regulation is sufficient to account for the observed difference in cytochrome *c* levels.

MATERIALS AND METHODS

Yeast Strains. D311-3A, D234-4D, B596, and B581 are haploid strains obtained from Fred Sherman and previously characterized (6). D311-3A is a *CYC1* wild-type strain. B596 and B581 are *cycl* nonsense mutants derived from D311-3A. B596 has an ochre mutation, *cycl-9*, in the second codon of the *cycl* gene, and B581 has an amber mutation, *cycl-76*, in the seventy-first codon. D234-4D is not related to the other three strains and contains a deletion of the entire *cycl* gene as well as flanking sequences (7). dRZ1 is a diploid strain used previously in studies of cytochrome *c* regulation (4).

Growth and [^{3}H]Adenine Labeling of Yeast Cells. Yeast cells were grown at 30°C with vigorous shaking in complete medium lacking adenine (3), with 2% raffinose for derepression. For the growth of the four haploid strains, the medium was supplemented with 0.01% Difco Bacto yeast extract and 0.02% Difco Bacto peptone. All cultures were maintained in exponential growth for at least 16 hr before labeling. For pulse-label experiments, cells were grown to a density of 2.7×10^7 cells per ml, then 200 μCi of [^{3}H]adenine (55 Ci/mmol, ICN Chemical and Radioisotope) was added to 2 ml of culture for the designated time (1 Ci = 3.70×10^{10} becquerels). Incorporation was stopped by the addition of 3 vol of ice-cold RNA extraction buffer (0.1 M Tris·HCl/0.1 M LiCl/0.1 mM EDTA, adjusted to pH 7.4).

For pulse-chase experiments, cells were grown to a density of 2.7×10^7 cells per ml, then 400 μCi of [^{3}H]adenine was added to 12 ml of culture. After 15 min of further growth, incorporation was stopped by the addition of 2 ml of adenine (25 mg/ml). Growth was continued, and 2-ml samples were removed at the specified times. Further growth of these 2-ml samples was prevented by addition of 3 vol of ice-cold RNA extraction buffer. Under these conditions, the incorporation of [^{3}H]adenine into trichloroacetic acid-precipitable radioactivity was effectively stopped by the addition of unlabeled adenine.

Cells grown on 5% glucose were labeled in the same manner, but higher specific activities were necessary because of lower levels of cytochrome *c* RNA found in these cells. Therefore, for pulse-labeling experiments 300 μCi of [^{3}H]adenine was added to 2 ml of culture, and for the pulse-chase experiments 500 μCi of [^{3}H]adenine was added to the 10 ml of culture.

Preparation of RNA. The 2 ml of labeled cells were washed twice with extraction buffer and resuspended in 0.5 ml of that buffer. Acid-washed glass beads (1 g, 0.45 mm diameter) were added, and the mixture was shaken in a Vortex mixer at 0°C for 2 min. Sodium dodecyl sulfate was added to a final concentration of 0.5%, and the RNA was deproteinized by phenol extraction (3). A typical yield from 2 ml of cells at 2.7×10^7 cells per ml was 60 μg of RNA with a specific activity of $1–4 \times 10^5$ cpm/μg depending upon the labeling conditions.

Preparation of DNA and DNA Filters. DNA of recombinant plasmid pYe*CYC1*(0.60) was used to assay for *CYC1* mRNA by hybridization. The sequence of the 609-base-pair yeast insert in this plasmid has been completely determined (8). pBR322 and pYe*CYC1*(0.60) DNA were prepared as described by Elwell *et al.* (9). Between 15 and 30 μg of DNA was loaded onto a 25-mm Millipore nitrocellulose filter (GSWP). DNA was diluted to 5 μg/ml in 0.1 M NaOH and heated at 67°C for 10 min. An equal volume of 0.05 M Tris·HCl (pH 7.9) and 0.1 M HCl was added and the pH was monitored with pH paper to ensure neutrality. The solution was then heated at 100°C for 5 min, cooled quickly by addition of 7 vol of ice-cold water, and brought to 5× NaCl/Cit (1× NaCl/Cit is 150 mM NaCl/15

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Abbreviation: NaCl/Cit, standard saline citrate (150 mM NaCl/15 mM sodium citrate).

mM sodium citrate) and 3 mM $MgCl_2$. The solution was then filtered through a filter that had been washed in 5× NaCl/Cit. The filter was dried at 80°C under reduced pressure for 3 hr.

Hybridization. A typical hybridization reaction was performed as follows. A 3-mm-diameter filter containing pBR322 DNA and a 3-mm-diameter filter containing pYe*CYC1*(0.60) DNA were added to a 0.5-ml solution of 40% formamide, 2× NaCl/Cit, 0.2% sodium dodecyl sulfate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and [^{3}H]RNA. This mixture was incubated at 40°C with gentle shaking for 64 hr. The filters were then washed at 40°C by shaking once with 2× NaCl/Cit and 40% formamide for 60 min, twice with 2× NaCl/Cit and 40% formamide for 15 min, 5 times with 2× NaCl/Cit for 5 min, and once with 95% ethanol. The filters were dried and the radioactivity bound to each filter was determined in a liquid scintillation counter.

In the initial experiments, the filters were treated with RNase A during the washing. However, this step had no effect on the radioactivity bound to the pBR322 filter and was discontinued.

The extent of RNA hybridization to the *cyc1* gene was determined by subtracting the cpm bound to the pBR322 filter from the cpm bound to the pYe*CYC1*(0.60) filter; usually between 40 and 100 cpm bound to the pBR322 filter and between 100 and 800 cpm bound to the pYe*CYC1*(0.60) filter. The counting efficiency in these experiments was 15%.

E. coli cells were grown and plasmid DNA was extracted in a P2 containment facility in compliance with the National Institutes of Health Guidelines pertaining to recombinant DNA experiments.

RESULTS

Specificity of Hybridization. The pYe*CYC1*(0.60) plasmid is a recombinant plasmid composed of the *E. coli* plasmid pBR322 joined to 609 base pairs of yeast DNA. The yeast DNA consists of 324 base pairs of the coding sequence of the *cyc1* gene, from the second codon through to the 3′ end, and 275 base pairs beyond the 3′ end of the gene (8). We wished to use this plasmid for RNA·DNA hybridization experiments to determine the rates of synthesis and levels of *cyc1* RNA in yeast cells. First, however, it was necessary to characterize the RNA that hybridized to pYe*CYC1*(0.60) DNA. For this purpose, [^{3}H]RNA was prepared from four haploid strains grown under derepressed conditions (on 2% raffinose) and incubated with pYe*CYC1*(0.60) DNA fixed to filters. The results of these hybridization experiments are presented in Table 1. D311-3A is a wild-type strain which gave a hybridization value, the difference in cpm bound to the pYe*CYC1*(0.60) filter compared to the pBR322 filter, defined as 100%. D234-4D is a *cyc1* mutant in which the yeast sequences present in the pYe*CYC1*(0.60) plasmid are deleted. As the results in Table 1 show, no detectable hybridization occurred between RNA from D234-4D and pYe*CYC1*(0.60) DNA. This result demonstrates that only RNA transcribed from the region of the yeast genome from which the pYe*CYC1*(0.60) yeast DNA was derived gives detectable hybridization to that plasmid. The other two strains, B596 and B581, are both *cyc1* nonsense mutants isolated from D311-3A. As can be seen in Table 1, these nonsense mutants contained less RNA homologous to the plasmid DNA than do wild-type cells. In addition, B596, which has a nonsense mutation at the second codon of the *cyc1* gene, contained less hybridizable RNA than did B581, which has the nonsense mutation at the seventy-first codon. This result is consistent with our previous finding that B581 cells have only half as much translatable *cyc1* mRNA as do wild-type cells (3). Because these two nonsense mutants are congenic to the wild-type strain, differing only in the point mutations in the *cyc1* gene, the different levels of hybridization of RNA from these three strains to pYe*CYC1*(0.60) DNA can be attributed only to differences in the levels of *cyc1* gene transcripts. Therefore, the results in Table 1 demonstrate that the *cyc1* gene transcript is the only RNA species that hybridizes at detectable levels to pYe*CYC1*(0.60) DNA.

Regulation of *cyc1* Gene Transcription. Studies on the rate of transcription of the *cyc1* gene were carried out with the diploid strain dRZ1. In Fig. 1, the extent of hybridization of labeled RNA from dRZ1 to the pYe*CYC1*(0.60) plasmid DNA fixed to filters is shown. From this curve, the saturation value for the DNA on filters was obtained. All subsequent experiments were performed with RNA concentrations below the saturation level so that DNA was always in excess. This saturation curve was determined for each batch of filters.

The rate of transcription of the *cyc1* gene relative to the rate of total transcription was measured by pulse-labeling cells with [^{3}H]adenine, isolating total RNA, then determining the fraction of labeled RNA that would hybridize to pYe*CYC1*(0.60) DNA on filters. One important consideration in these experiments was that the labeling time be sufficiently short that the rate of accumulation of radioactivity in *cyc1* RNA be equal to the rate of its transcription because, at long labeling times, the specific activity of an mRNA approaches a steady state at which radioactivity is lost through degradation as fast as it is gained through new synthesis. To ensure that we were measuring synthesis of *cyc1* RNA and not the steady-state level, the relative specific activity of *cyc1* RNA was compared to the specific activity of total RNA (mostly stable RNA species) for three different labeling times, 2, 5, and 10 min. If a steady state were reached within 10 min, the increase in the relative specific activity of *cyc1* RNA would be less over the three time points than the increase in specific activity of total RNA. (Because the concentration of *cyc1* RNA could not be measured directly, the relative specific activity, cpm in *cyc1* RNA per μg of total RNA,

Table 1. Hybridization of RNA from *cyc1* mutants

Strain	RNA added, μg	RNA specific activity × 10^{-5}	cpm pBR322 filter	cpm pYe*CYC1* (0.60) filter	cpm hybridized*	Fraction cpm hybridized × 10^5	Wild-type hybridization, %
D311-3A	11.65	1.69	105	408	303	15.4	100
D234-4D	35.6	2.34	88	81	—	—	0
B596	57	3.68	46	196	150	0.72	5
B581	13.6	1.8	71	197	126	5.2	34

Cells were grown exponentially over at least 16 hr in medium containing 2% raffinose. A 2-ml sample was labeled at a density of 2.7×10^7 cells per ml with 100 μCi of [^{3}H]adenine for 30 min.

* The values for cpm hybridized were calculated by substracting the cpm bound to the pBR322 filter from those bound to the pYe*CYC1*(0.60) filter.

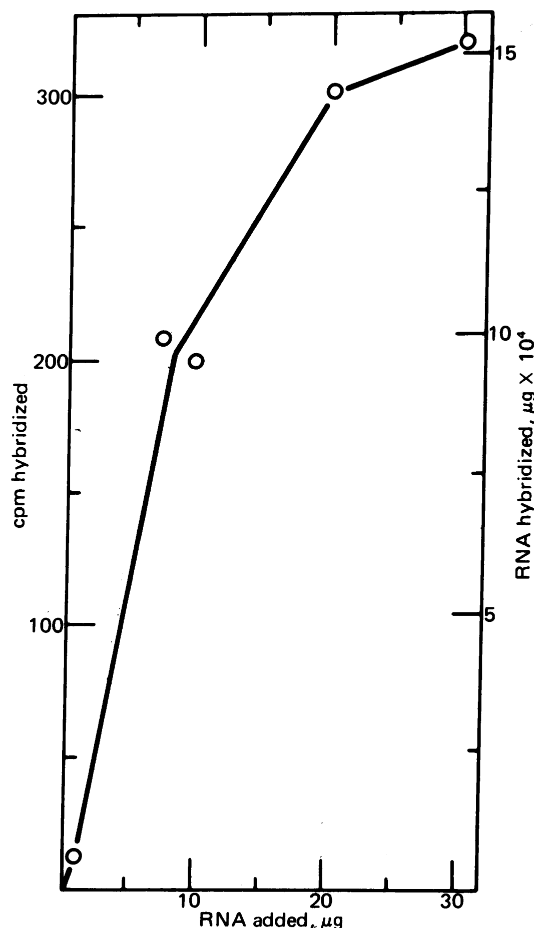

FIG. 1. Hybridization of dRZ1 [^{3}H]RNA to plasmid DNA on filters. Cells of strain dRZ1 were grown in medium containing 2% raffinose and labeled with [^{3}H]adenine for 10 min. The specific activity of the RNA was 2.14×10^5 cpm/µg. The values for "cpm hybridized" were determined by subtracting the cpm bound to the pBR322 filter from the cpm bound to the pYe*CYC1* (0.60) filter. The values for "µg RNA hybridized" were obtained by dividing the cpm hybridized by the specific activity of the RNA.

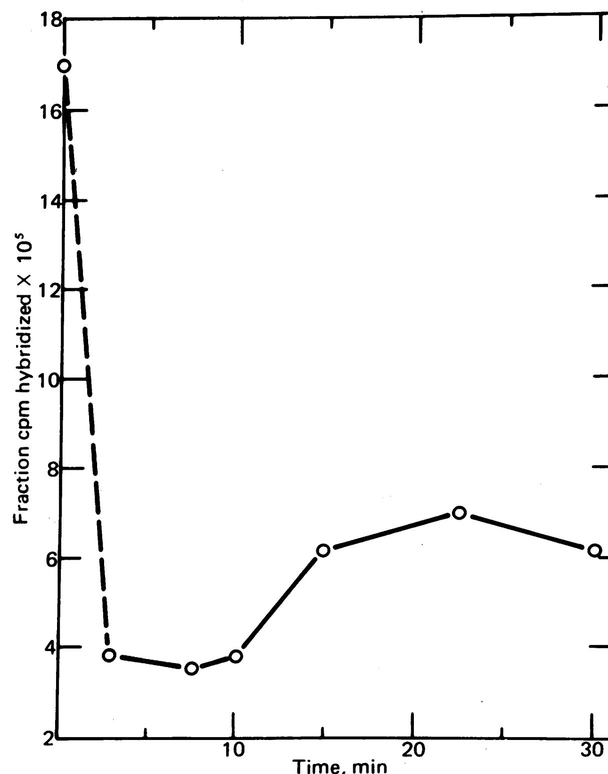

FIG. 2. Kinetics of repression of *cyc1* RNA synthesis. Cells of strain dRZ1 were grown for 16 hr in medium containing 2% raffinose to a cell density of 2.7×10^7/ml. A 2-ml sample was labeled with [^{3}H]adenine for 2 min. An equal volume of medium containing 10% glucose was added to the remaining culture and, at various times afterwards, 4-ml samples were labeled with 400 µCi of [^{3}H]adenine for 2 min. RNA was prepared from the samples and the fraction of total incorporated [^{3}H]adenine that hybridized specifically to *cyc1* DNA was determined. Time zero was the time of glucose addition.

was used.) The results are presented in Table 2. The data clearly show that a steady state was not reached within 10 min; the relative specific activity of *cyc1* RNA (column 4) increased at the same rate as the specific activity of total RNA (column 3) over the 10 min, as indicated by the constant ratio of the value in column 4 divided by that in column 3 over the 2-, 5-, and 10-min labeling periods (column 5). This ratio, the fraction of incorporated [^{3}H]adenine that hybridized specifically to pYe*CYC1*(0.60) DNA, represents the relative rate of transcription of the *cyc1* gene. As seen in Table 2, this fraction is 6 times higher for RNA from derepressed cells grown on raffinose as compared to that from repressed cells grown on glucose. We conclude, therefore, that the expression of the *cyc1* gene is regulated at the level of transcription.

Kinetics of Repression of *cyc1* Gene Expression. We reported previously that the rate of cytochrome *c* protein synthesis and the level of translatable *cyc1* RNA decreased from the derepressed to the repressed values with a 2-min half-time upon addition of 5% glucose to a derepressed culture (4). In this present study, to determine if this rapid repression were due to a repression of transcription, we measured the rate of *cyc1* RNA synthesis by hybridization of RNA pulse-labeled with [^{3}H]adenine at various times after glucose addition to a derepressed culture. The results, presented in Fig. 2, show that the rate of transcription of the *cyc1* gene falls from the derepressed to the repressed level within 2.5 min, the first time point after glucose addition. Fifteen minutes after this initial repression of *cyc1* RNA synthesis, there was a transitory increase in the rate

Table 2. Rate of transcription of the *cyc1* gene

(1) Carbon source	(2) Length of labeling time, min	(3) Specific activity of total RNA, cpm × 10^{-5}/µg	(4) cpm hybridized/ µg RNA added	(5) Fraction of cpm hybridized × 10^5
2% raffinose	2	0.58	9.52	16.4
	5	1.22	20.5	16.8
	10	1.71	28.5	16.7
5% glucose	2	0.63	1.66	2.63
	5	1.08	2.91	2.70
	10	1.41	3.56	2.52

of *cycl* gene transcription to twice the fully repressed rate. The reason for this increase is not known, but it does not result in the accumulation of functional cytochrome *c* mRNA. In experiments using a radioimmunoassay that was sensitive to both iso-1 and iso-2 (the minor species) cytochromes *c*, no transitory increase in the *in vivo* rate of cytochrome *c* protein synthesis was observed after the rapid glucose repression of a derepressed culture (4).

Half-Life of *cycl* RNA. The half-life of *cycl* RNA was determined by labeling cells with [^{3}H]adenine for 15 min, then preventing further incorporation of label by the addition of excess unlabeled adenine. RNA was extracted at various times

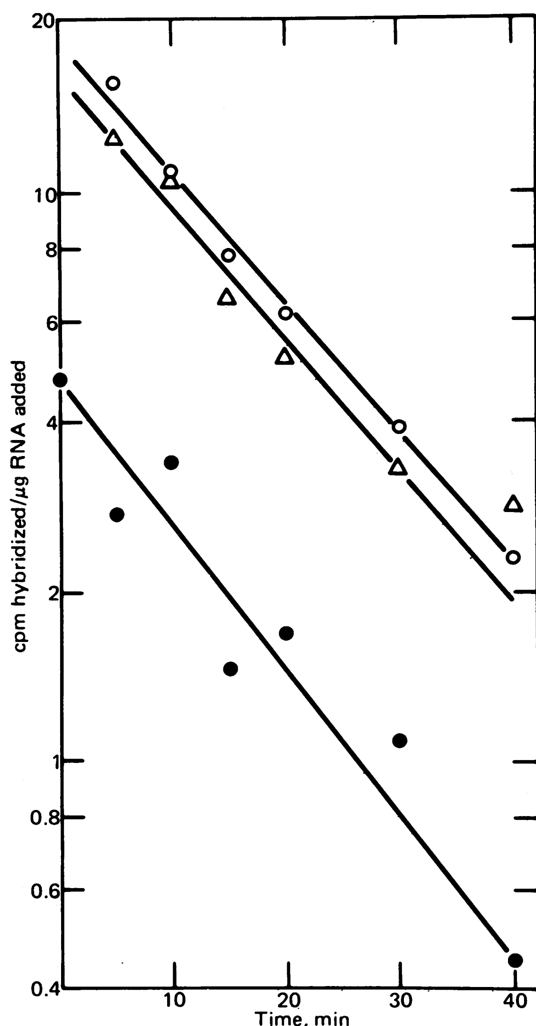

FIG. 3. Half-life of *cycl* RNA. Cells of strain dRZ1 were grown in medium containing either 2% raffinose (○) or 5% glucose (●) and labeled with [^{3}H]adenine for 15 min. Incorporation of label was stopped by addition of unlabeled adenine, and cell growth was continued. At various times after incorporation was stopped, 2-ml samples were removed, RNA was prepared, and the radioactivity remaining in *cycl* RNA was determined by hybridization. In one case (△), cells were grown and labeled in medium containing 2% raffinose, and then glucose was added to a final concentration of 5% along with the unlabeled adenine. The specific activities of the RNA prepared were 1.4×10^5 cpm/μg (○), 1.7×10^5 cpm/μg (●), and 1.32×10^5 cpm/μg (△). Time zero is the time at which incorporation of [^{3}H]-adenine was stopped. The data are plotted as the cpm remaining in hybridizable RNA per μg of RNA added and are not corrected for differences in the initial specific activities of the RNA. Therefore, the ordinate intercepts cannot be directly compared to obtain the relative levels of *cycl* RNA in these cells in the same way that the fraction of hybridizable radioactivity can.

during the continued growth of the cells, and the radioactivity remaining in *cycl* RNA was determined by hybridization. The results of these experiments are presented in Fig. 3, a plot of the logarithm of the radioactivity remaining in hybridizable RNA per μg of RNA added against time. The half-life of *cycl* RNA was essentially the same under repressed (12 min) and derepressed (13.5 min) conditions. These results are consistent with the results of the pulse-labeling experiments presented in Table 2. If the half-life of *cycl* RNA were much shorter than 10 min, then the relative specific activity of *cycl* RNA would not have increased over the 10-min labeling period.

The half-life of *cycl* RNA was also determined in cells undergoing repression. Cells grown under derepressed conditions were labeled for 15 min with [^{3}H]adenine. Unlabeled adenine and glucose were added simultaneously, and samples of culture were removed at subsequent times. RNA was prepared, and the radioactivity remaining in *cycl* RNA was determined by hybridization. Fig. 3 shows that *cycl* RNA had the same half-life, 13 min, during repression as it did in fully repressed or derepressed cells.

DISCUSSION

The results presented here clearly demonstrate that the expression of the *cycl* gene is under transcriptional regulation. Cells grown under derepressed conditions synthesize *cycl* RNA at a 6-fold greater rate than do cells grown repressed in medium containing glucose. This 6-fold difference equals that observed for the rate of cytochrome *c* protein synthesis in derepressed as compared to repressed cells and closely approximates the 4-fold difference observed in the levels of translatable *cycl* mRNA between derepressed and repressed cells (3). Therefore, regulation of *cycl* gene transcription is sufficient to account for the differences in the levels of cytochrome *c* protein observed in derepressed and repressed cells. Recently, the *ura3* gene of yeast has been shown, by hybridization experiments to pulse-labeled RNA, to be under transcriptional control (10). The half-life of this *ura3* RNA was reported to be 10.5 min, very close to the half-life of the *cycl* RNA.

One interesting difference between the results presented here and those reported previously relates to the half-life of *cycl* RNA. As measured by hybridization, the half-life is 13 min. However, this degradative rate is too slow to account for the 2-min half-time of repression of cytochrome *c* protein synthesis upon glucose addition to derepressed cultures that we reported previously (4). Even with the immediate repression of transcription (Fig. 2), the rate of cytochrome *c* protein synthesis could not decrease faster than the half-life of the functional mRNA, which was observed to decrease from the derepressed to the repressed levels upon glucose addition at the same rate as the 2-min half-time of repression of protein synthesis. This finding is not unprecedented; similar differences in functional and hybridizable half-lives of mRNA have been reported in prokaryotic systems (11, 12). This apparent contradiction would be resolved if the degradation of mRNA were to proceed by an initial rapid endonucleolytic cleavage of the RNA, followed by degradation of the remaining fragments. The first internal break in the mRNA would render it untranslatable, while the fragments remaining would still hybridize to DNA on filters.

An interesting, and as yet unexplained, observation made here and previously (3) is that nonsense mutants have lowered levels of *cycl* RNA, as determined by both hybridization and *in vitro* translation. As compared to wild type, B581 contained half the level of translatable *cycl* mRNA and one-third the level of hybridizable *cycl* RNA, whereas B596 contained only 5% as much hybridizable RNA as did the parental wild type. The reason why either synthesis or stability of the *cycl* RNA is di-

minished in cells carrying these nonsense mutations is not known. Nevertheless, the absence of mRNA in the nonsense mutations is useful in identification of transcription products specified by the *cyc1* gene.

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