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Transcriptional Regulation of the *Kluyveromyces lactis* β-Galactosidase Gene

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We examined the molecular basis for β-D-galactosidase (EC 3.2.1.23) induction in the yeast *Kluyveromyces lactis*. The protein synthesis inhibitor anisomycin effectively blocked both protein synthesis and enzyme induction by lactose. Further, hybridization analysis with the cloned β-galactosidase gene indicated coordinate increases in the concentration of β-galactosidase messenger ribonucleic acid and enzyme activity. The half-life of β-galactosidase messenger ribonucleic acid was the same (4.8 ± 0.4 min) when measured both before and at succeeding times during enzyme induction. These results strongly support the hypothesis that expression of the yeast β-galactosidase gene is subject to transcriptional regulation.

The yeast *Kluyveromyces* (*Saccharomyces* [26]) *lactis* can utilize lactose as its sole carbon and energy source. In this species lactose is hydrolyzed by an intracellular β-D-galactosidase (EC 3.2.1.23) which is induced by its substrate and related compounds (9, 10, 23). The mechanism(s) regulating this induction is not known. Since yeasts offer the opportunity to examine by both genetic and biochemical techniques the molecular details of gene regulation in eucaryotes, we have used multiple approaches to study the mechanism(s) by which lactose induces β-galactosidase in *K. lactis* (8, 10, 20).

Our objective in this study was to determine whether enzyme induction was the result of post-translational, translational, or transcriptional regulation. To distinguish between post-translational regulation and translational or transcriptional regulation, the effect of a protein synthesis inhibitor on enzyme induction was examined. When simultaneous inhibition of protein synthesis and enzyme induction indicated that post-translational regulation was not involved, the role of transcriptional regulation was studied. Using the cloned β-galactosidase gene (9), we tested whether an increase in the concentration of β-galactosidase messenger ribonucleic acid (mRNA) could account for enzyme induction. Further, we examined the half-life of β-galactosidase mRNA both before and during enzyme induction to determine whether mRNA stabilization might affect enzyme induction.

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MATERIALS AND METHODS

Strain, media, and growth conditions. The wild-type strain of *K. lactis* used in this study, Y1140, has been described previously (20). Cells were grown in either defined sulfate medium or yeast nitrogen base with amino acids medium (Difco Laboratories, Detroit, Mich.). Defined sulfate medium at pH 5.5 was prepared as previously described (9). Yeast nitrogen base with amino acids medium was prepared at twice the manufacturer's suggested concentration. Carbon sources were added to a final concentration of 20 mM. All cell cultures were grown at 30°C as previously described (20). Cell growth was monitored spectrophotometrically by absorption at 600 nm (A_{600}). One A_{600} unit was equal to 3.7×10^7 cells per ml. Under all growth conditions, cultures entered the stationary growth phase between 9 and 12 A_{600} units. All experiments were conducted with logarithmic-phase cells at an A_{600} of less than 6.

Preparation of cell-free extracts. Logarithmic-phase cells were harvested by centrifugation, suspended in cold extraction buffer (0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5; 0.1 M LiCl; and 1 mM ethylenediaminetetraacetate) at a concentration of 25 to 50 A_{600} units/ml, mixed with an equal volume of 0.5-mm-diameter glass beads (B. Braun Instruments, San Francisco, Calif.) and broken by blending in a Vortex mixer 10 times for 20 s. The cell lysate was decanted from the glass beads and then clarified by centrifugation for 20 min at 10,000 × *g*. The supernatant solution was stored at 4°C and assayed for β-galactosidase activity within 24 h.

Inhibition of protein synthesis. Cells were grown overnight in defined sulfate medium containing 20 mM sorbitol. They were diluted to an A_{600} of 0.75 in fresh medium and allowed to grow to 1.0 A_{600} units/ml. At this time [^{14}C]leucine (30 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was added at

1 μCi/ml. Thirty minutes later the culture was divided and induced with 20 mM lactose in either the presence or the absence of 50 μg of anisomycin per ml (Pfizer, Inc., Groton, Conn.). Protein synthesis inhibition was monitored as incorporation of labeled amino acid into acid-precipitable material. Samples (25 μl) were taken at regular intervals for 30 min before and 145 min after induction, precipitated with 5% trichloroacetic acid containing 1 μg of leucine per ml, collected on Whatman GF/C filters by filtration, and washed with 95% ethanol. Radioactivity on dried filters was determined in a liquid scintillation counter.

To measure the effect of anisomycin on the induction of β-galactosidase activity, the procedure described above was modified slightly. Stationary-phase cells were diluted into fresh defined sulfate medium and grown from 0.2 to 0.35 A_{600} units/ml. The culture was then divided and induced with 20 mM lactose with or without 50 μg of anisomycin per ml. β-Galactosidase activity in whole cells was monitored as described below.

Preparation of total RNA. Logarithmic-phase cells were chilled by the addition of 500 g of crushed ice per liter of culture and harvested by centrifugation. For each 1 g of cells, 5.5 ml of extraction buffer was added. The cell suspension was mixed with glass beads and broken in a Braun homogenizer as previously described (8). The supernatant was rapidly decanted into 0.5 volume of buffer-saturated phenol prewarmed to 55°C. RNA was deproteinized for 2 min at 55°C with vigorous shaking, and then 0.5 volume of chloroform was added and shaking was continued for 10 min at 35°C. After repetition of the phenol-chloroform extraction, the RNA was extracted twice at 35°C with an equal volume of chloroform. The RNA was precipitated with 1 volume of cold 4 M LiCl for 2 h at 4°C, collected by centrifugation for 20 min at 10,000 × *g*, washed twice with 2 M LiCl, and suspended in sterile water. The RNA was precipitated two additional times in 0.2 M NaCl by addition of 2 volumes of cold ethanol and lyophilized before suspension in liquid hybridization buffer at a concentration of 5 to 10 mg/ml. The average yield from 200 A_{600} units of cells was 1 mg of RNA.

Labeling and extraction of RNA. RNA was labeled with [^{3}H]adenine (15.5 Ci/mmol, New England Nuclear). Log-phase cells at a density of 3.0 A_{600} units/ml were labeled with 30 μCi of [^{3}H]adenine per ml. After 5 min of labeling, further incorporation of label was stopped within 1 min by the addition of unlabeled adenine to a final concentration of 50 μg/ml (data not shown). Samples (5 ml), removed at specified times, were chilled immediately by dilution into 3 volumes of ice-cold extraction buffer. Cells were collected by centrifugation, washed with buffer, resuspended in extraction buffer at a density of 20 A_{600} units/ml, and broken by blending in a Vortex mixer with glass beads. RNA was isolated from these lysates by phenol-chloroform extraction as described by Zitomer and Hall (27). A typical yield from 15 A_{600} units of cells was 150 μg of RNA, with a specific activity of 2×10^4 to 5×10^4 cpm/μg. The specific activity of the RNA isolated from each time point was constant during the chase period of these experiments, indicating no detectable turnover of RNA (data not shown).

Preparation of DNA, [^{32}P]DNA, and DNA filters. pBR322 and pKl6 plasmid deoxyribonucleic acids (DNAs) were isolated by cesium chloride-ethidium bromide density gradient centrifugation as previously described (9). A specific DNA fragment from the recombinant plasmid pKl6 (9) was used to assay for β-galactosidase mRNA by hybridization. This hybridization probe was isolated after digestion of pKl6 with the restriction nucleases *Pvu*I, *Xho*I, and *Sal*I (New England Biolabs, Beverly, Mass., or Bethesda Research Laboratories, Rockville, Md.) and fractionation of the restriction fragments by agarose gel electrophoresis (11). A 4.7-kilobase DNA fragment, containing approximately 3.3 kilobases comprising the β-galactosidase gene, was isolated by the hydroxyapatite procedure of Tabak and Flavell (22). The DNA fragment was labeled with [^{32}P]deoxyadenosine 5′-triphosphate (300 to 500 Ci/mmol; New England Nuclear) by "nick translation" (17), with slight modification as described previously (9). Specific activities of 2×10^7 to 4×10^7 cpm/μg were obtained.

To prepare DNA-containing filters, plasmid DNAs were linearized by digestion with *Eco*RI (pBR322) or *Pvu*I (pKl6) as previously described (7, 9), and then 15 to 20 μg of DNA was loaded onto 25-mm-diameter BA85 nitrocellulose filters (Schleicher & Schuell Co., Keene, N.H.) under denaturing conditions (2, 14) as described by Zitomer et al. (28). After drying for 2.5 h at 80°C under reduced pressure, 5-mm-diameter microfilters containing 1 to 2 μg of DNA were prepared.

RNA excess hybridization to [^{32}P]DNA. β-Galactosidase probe [^{32}P]DNA was hybridized with excess RNA under conditions similar to those described by Casey and Davidson (6), which favor formation and stability of RNA-DNA hybrids over DNA-DNA hybrids and which result in no detectable RNA degradation. Samples in 75% formamide–0.4 M NaCl–0.01 M ethylenediaminetetraacetate–0.01 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) were incubated at 49.5°C. This incubation temperature was chosen after preliminary experiments showed that RNA-DNA hybrid formation at this temperature proceeded without DNA probe renaturation. Reactions of 300 μl contained approximately 30 ng of labeled DNA (1×10^5 to 2×10^5 cpm) per ml and concentrations of RNA from 0.5 to 10 mg/ml depending upon the expected concentration of β-galactosidase mRNA. Control experiments showed that all reactions contained excess RNA and reached similar hybridization maxima (data not shown). Reactions were overlaid with mineral oil to prevent evaporation, and the double-stranded probe was denatured by heating for 5 min at 92°C. Duplicate 10-μl samples were removed with prewarmed micropipettes at times appropriate for obtaining desired R_0t values (R_0t is defined as the product of the concentration of RNA in moles of nucleotides per liter and time in seconds). Samples were diluted into 1 ml of cold assay buffer (0.03 M sodium acetate, 0.05 M NaCl, 1 mM $ZnSO_4$, and 5% glycerol, pH 4.6, containing 20 μg of denatured salmon sperm DNA per ml), and portions were assayed to determine the trichloroacetic acid-precipitable radioactivity before and after treatment with excess nuclease S1. Nuclease digestion and analysis of precipitable radioactivity were performed as described by

McKnight and Schimke (18), except that Enzo GF/C filters were used and radioactivity was counted without solubilization of filters. Results are presented as percentages of the maximum hybridization measured in each reaction. A background of 3 to 5% of the input counts was measured in control reactions containing hen oviduct RNA or no RNA. When this background was subtracted, maximum hybridization averaged 20% of the input counts. Since this DNA probe is double stranded and contains sequences other than those coding for β-galactosidase mRNA, the maximum hybridization expected under optimum labeling and hybridization conditions would be approximately 35% of the input counts.

Filter hybridization. Filter hybridization of [^{3}H]RNA to both pBR322- and pKl6-containing filters was performed under conditions similar to those described by Zitomer et al. (28). In our experiments, however, the [^{3}H]RNA in the reaction was denatured by heating the reaction mixture for 10 min at 80°C, incubation of the reaction was for 40 to 42 h, and ribonuclease A treatment was included. Saturating concentrations of *K. lactis* [^{3}H]RNA were determined for each batch of pKl6-containing filters. In all subsequent half-life analyses, DNA was always in excess (data not shown).

The extent of RNA hybridization was determined by subtracting counts per minute bound to the pBR322-containing filter from the counts per minute bound to the pKl6-containing filter; usually 35 to 40 cpm bound to the pBR322 filter and between 100 and 600 cpm bound to the pKl6 filter. The specific activity of each RNA preparation was used to calculate the nanograms of sequences hybridized per 50 μg of total RNA.

Miscellaneous procedures. β-Galactosidase activity was assayed in whole cells and cell-free extracts as previously described by Sheetz and Dickson (20). Protein concentrations were measured according to the method of Lowry et al. (16), using ovalbumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. The concentrations of RNA and DNA were measured spectrophotometrically at A_{260} (1 A_{260} unit was equal to 50 μg of nucleic acid per ml).

The single-strand nuclease S1, prepared by the standard procedure of Vogt (25), was kindly supplied by E. James of the Department of Biochemistry, University of Kentucky, Lexington.

Formamide (reagent grade; Fisher Scientific Co., Pittsburgh, Pa.) was recrystallized three times (6) for use in liquid hybridizations.

Biohazard considerations. All experiments involving recombinant DNA were conducted under P2 containment conditions according to the National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Induction of β-galactosidase requires protein synthesis. The role of protein synthesis in the induction of enzyme activity was tested with the drug anisomycin. This inhibitor of protein synthesis was chosen because of the known resistance of *K. lactis* to the more commonly used inhibitor cycloheximide (21). When added to cells in logarithmic growth, anisomycin inhibited protein synthesis to less than 10% of control levels within 15 min (Fig. 1A). Further, simultaneous addition of drug and the inducer lactose resulted in complete inhibition of enzyme induction (Fig. 1B). The effect of anisomycin on enzyme induction supports the hypothesis that regulation of enzyme induction occurs at the transcriptional or translational level rather than post-translationally.

β-Galactosidase mRNA level varies coordinately with the level of β-galactosidase activity. The increased levels of β-galactosidase activity and enzyme protein (8; unpublished data) in induced cells probably reflect an increased concentration of β-galactosidase mRNA. This possibility was examined by using the cloned β-galactosidase gene (9) as a probe to measure β-galactosidase mRNA levels in RNA excess hybridization reactions under conditions favoring RNA-DNA hybrids. The radioactive probe was a 4.7-kilobase DNA fragment of the plasmid pKl6 whose ends were defined by the *Xho*I and *Sal*I sites at map coordinates 2.8 and 6.35 (7). Approximately 3.3 kilobases of this DNA fragment comprise the β-galactosidase gene, whereas 0.52 kilobases are from pBR322 and the remaining 0.9 kilobases consist of other yeast sequences which do not hybridize to yeast RNA (R. C. Dickson, unpublished data). Thus, the DNA fragment is a specific probe for β-galactosidase sequences. Total RNA used in the hybridizations was isolated from wild-type cells before and at various times after addition of the inducer lactose. The level of enzyme induction was measured by assaying β-galactosidase specific activity in cell-free extracts prepared in parallel with RNA preparations and is expressed relative to the β-galactosidase specific activity measured in uninduced cells.

The hybridization data demonstrated that the concentration of β-galactosidase mRNA in wild-type cells increased after induction (Fig. 2). Cells induced 2.0-, 4.5-, 13.2-, and 20-fold for β-galactosidase activity clearly had more β-galactosidase mRNA than did uninduced cells. To determine quantitatively the R_0t value for half-maximal hybridization of each RNA ($R_0t_{1/2}$), the data were replotted and analyzed by the double reciprocal method described by Bishop (3). The $R_0t_{1/2}$ values varied coordinately with the increases in β-galactosidase activity (Table 1). It was apparent from these data that the concentration of β-galactosidase mRNA sequences increased after induction and that the level of increase was paralleled by increased β-galactosidase activity.

Half-life of β-galactosidase mRNA is unchanged during induction. An increase in the

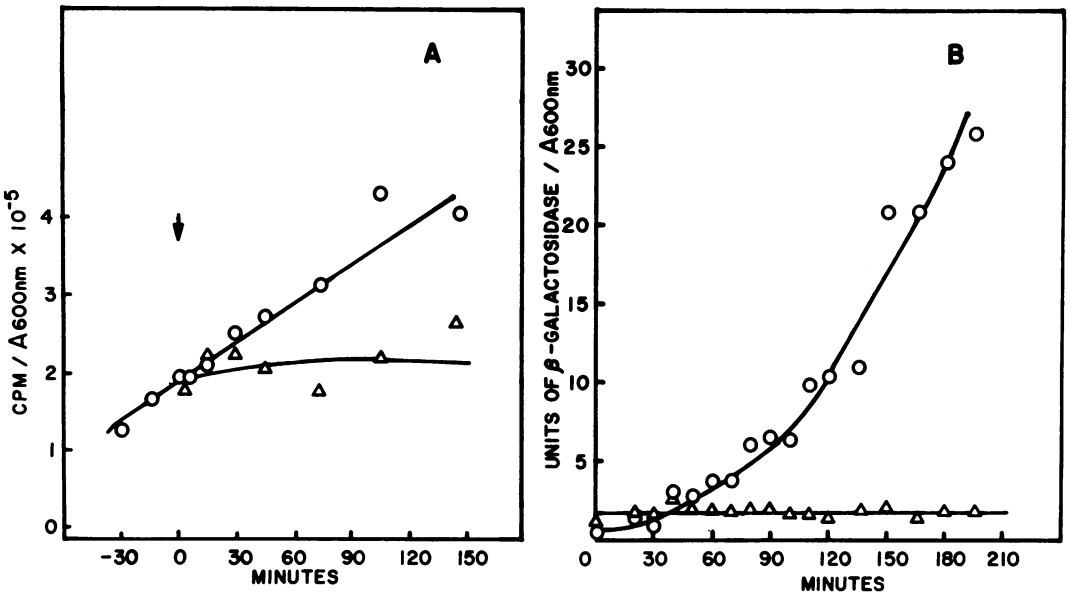

FIG. 1. *Inhibition of protein synthesis and enzyme induction by anisomycin. (A) Protein synthesis monitored in K. lactis induced in the presence* (△) *or absence* (○) *of 50 μg of anisomycin per ml; a single culture was prelabeled with [^{14}C]leucine, divided (arrow), and induced with 20 mM lactose. (B) Enzyme induction in K. lactis by 20 mM lactose in the presence* (△) *or absence* (○) *of 50 μg of anisomycin per ml. Details are given in the text.*

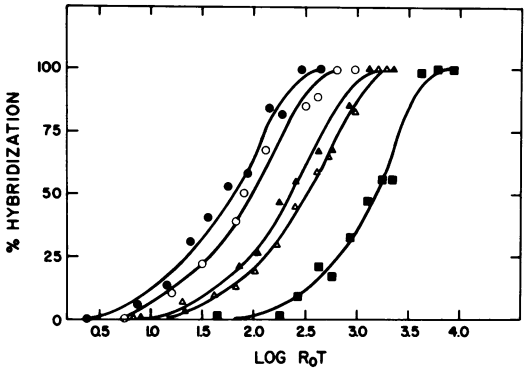

FIG. 2. *Concentration of β-galactosidase mRNA sequences. The relative concentration of β-galactosidase mRNA in K. lactis was determined by measuring the rate of hybridization of a radioactive β-galactosidase gene probe to total cellular RNA. Reactions contained total RNA isolated from uninduced cells* (■), *and from cells induced 2* (△)-, *4.5* (▲)-, *13.2* (○)-, *and 20* (●)-*fold for β-galactosidase activity. Details are given in the text.*

TABLE 1. *Relative concentration of β-galactosidase-specific mRNA sequences in K. lactis*

Culture	Enzyme induction level	$R_0t_{1/2}$	β-Galactosidase mRNA level[a]
Uninduced	1.0	1,190	1.0
Induced	2.0	640	1.86
Induced	4.5	280	4.25
Induced	13.2	90	13.14
Induced	20.0	58	20.5

[a] $R_0t_{1/2}$ uninduced/$R_0t_{1/2}$ induced.

concentration of β-galactosidase mRNA may occur in several ways, for example, by an increase in the rate of transcription or, alternatively, by a decrease in the rate of degradation. Since it was not technically feasible to measure the first possibility, we measured the rate of β-galactosidase mRNA degradation. The half-life of β-galactosidase mRNA was determined by labeling cells with [^{3}H]adenine for 5 min and then preventing further incorporation of label by the addition of excess unlabeled adenine. Preliminary experiments established that within 1 min the excess unlabeled adenine blocked further incorporation of [^{3}H]adenine (data not shown). RNA was extracted at various times during continued growth of the cells, and the radioactivity remaining in β-galactosidase mRNA was determined by filter hybridization. In three experiments, the half-life of β-galactosidase mRNA was the same, 4.8 ± 0.4 min, for both uninduced and induced cells. The results of a typical experiment are shown in Fig. 3. We conclude that the increased level of β-galactosidase mRNA is not due to a decreased rate of degradation, but rather that it probably results from increased initiation of transcription.

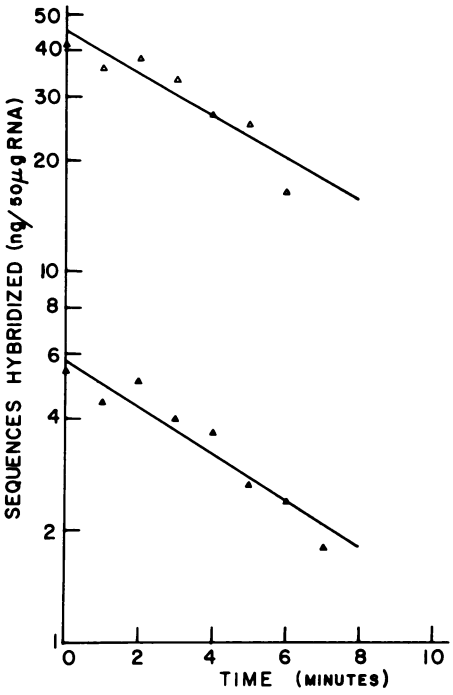

FIG. 3. *Half-life of β-galactosidase mRNA. In uninduced (▲) or induced (△) cultures of K. lactis pulse-labeled with [^{3}H]adenine, the decay of radioactivity remaining in β-galactosidase mRNA was determined by filter hybridization to pKl6 DNA. Background hybridization to control filters was subtracted, and data were corrected for the specific activity of the RNA. Details are given in the text.*

DISCUSSION

We have analyzed the molecular basis for lactose induction of β-galactosidase activity in *K. lactis*. Experiments were designed to differentiate among post-translational, translational, and transcriptional regulation. Analysis of the effect of protein synthesis inhibition indicated that lactose was ineffective in inducing β-galactosidase activity in the absence of protein synthesis. This result suggested that post-translational regulation was not involved in the induction of β-galactosidase; i.e., induction does not proceed by activation of an inactive β-galactosidase precursor.

Isolation of the cloned β-galactosidase gene (9) made possible hybridization experiments to test directly whether regulation of β-galactosidase gene expression occurs at the level of transcription. RNA-DNA hybridization analysis demonstrated increased concentrations of β-galactosidase mRNA during lactose induction of enzyme activity, the concentration of β-galactosidase mRNA varying coordinately with the level of β-galactosidase activity. From this result we hypothesized that expression of the β-galactosidase gene was under transcriptional regulation. Analysis of the chemical half-life of β-galactosidase mRNA as measured by hybridization to a cognate DNA indicated that the increase in β-galactosidase mRNA concentration was probably due to increased initiation of transcription rather than a decreased rate of degradation, since the rate of β-galactosidase mRNA degradation was the same in uninduced and induced cells. Because by our methods we examined only the chemical half-life of β-galactosidase mRNA, we cannot exclude the possibility that the functional or synthetically active half-life of this mRNA might differ in uninduced versus induced cells. For example, induction could increase the synthetic half-life of β-galactosidase mRNA without changing its chemical half-life. This would lead to an increase in enzyme activity, as observed, but it would not account for the observed increase in β-galactosidase mRNA. Currently, there is no evidence in yeasts for disparities between chemical and functional half-lives.

In *Saccharomyces cerevisiae*, regulation of several genes (orotidine-5'-phosphate carboxylyase [1], iso-1-cytochrome *c* [28], the *GAL1 GAL7 GAL10* gene cluster [12], allophanate hydrolase [15], arginase [4], and ornithine carbamoyltransferase [19]) has been demonstrated to operate, at least in part, at the level of transcription. The half-lives of five of these mRNA's have been determined (1, 4, 15, 19, 28), and the half-life of β-galactosidase (4.8 min) is most similar to the short half-lives (3 to 7 min) reported for allophanate hydrolase, arginase, and ornithine carbamoyltransferase. Interestingly, all of these half-lives, whether determined by measuring chemical half-lives (β-galactosidase; 1, 28) or the synthetic capacity half-lives (4, 15, 19) of the mRNA's are shorter than the 18 to 30 min reported for global synthetic capacity half-lives (5, 13, 24). Perhaps, as suggested by Bossinger and Cooper (4), global synthetic capacity half-lives represent average values weighted towards decay rates of RNA species accounting for the bulk of the mRNA pool and are not representative of the more labile mRNA's specifying proteins which are induced or repressed in response to a changing environment. Our data for β-galactosidase mRNA support this hypothesis.

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