Effect of Chemically-Induced, Cloned-Gene Expression on Protein Synthesis in *E. coli*

Thomas K. Wood and Steven W. Peretti*
Department of Chemical Engineering, North Carolina State University, Raleigh, North Carolina 27695-7905

Received August 28, 1990/Accepted January 15, 1991

Earlier experiments in our lab investigated the metabolic limitations of cloned-gene expression in bacterial cells (for over-production of β-lactamase). These experiments showed that the steady-state concentration of ribosomal RNA decreased upon plasmid amplification while both the synthesis rate and steady-state β-lactamase mRNA level increased significantly. This appeared to indicate substantial limitations exist within the translational machinery of the bacterial cell at high copy numbers. To establish the generality of this phenomenon, the impact of increasing protein expression from a plasmid by chemically inducing a strong promoter while maintaining constant copy number has been investigated. A plasmid has been constructed which contains the *lacZ* gene under control of the *tac* promoter and contains the *parB* stability locus to maintain plasmid stability. Using this vector, β-galactosidase expression in chemostat cultures operated at specific growth rates of 0.6 h^{-1} was induced with IPTG such that enzyme activity was varied over a 460-fold range. When fully induced, β-galactosidase protein production represented 14 wt % of total cell protein. As transcription was induced, the synthesis rate of the β-galactosidase mRNA increased 42-fold while the steady-state level of β-galactosidase mRNA increased only fourfold. This indicates mRNA stability may play a larger role for β-galactosidase expression with a strong promoter than seen with β-lactamase production in the elevated copy number system. Furthermore, rRNA synthesis rates increased at high expression rates as seen in the copy number experiments. However, unlike the amplified-plasmid system, the steady-state levels of rRNA increased as well. Since the total protein levels closely followed the steady-state level of rRNA, translational limitations are again suggested for the chemically induced-transcription system.
Key words: Escherichia coli • protein synthesis • metabolic limitations • cloned-gene expression • β-galactosidase

INTRODUCTION

As the biotechnology market matures, there is an increasing incentive to determine at the metabolic level what limits protein production within recombinant hosts. After delineating these mechanisms, recombinant protein production can be enhanced through appropriate cultivation strategies and genetic manipulations.

Recent studies have shown that the stability of the mRNA is important in cloned-gene expression. Chan et al.[4] have shown that chloramphenicol acetyltransferase (CAT) production can be increased five to eightfold by increasing the stability of its mRNA. The *cat* structural gene was cloned into the intron region of the very stable primary transcript of the *td* gene of phage T4, and the stability of the *cat* mRNA increased by a factor of 2. The flanking T4 intron regions are 8- to 22-fold more stable than the average *E. coli* mRNA, and they appear to stabilize the *cat* transcript due their highly-ordered RNA structure.[4] It appears the stability of the cloned-gene mRNA may limit productivity when its half-life is relatively short.

High expression of a cloned-gene may also be limited by the translational machinery of the cell. Nomura et al. have shown that for nonrecombinant *E. coli* systems, mRNA levels appear to be in excess.[21] Using an RNA polymerase overproduction system, they found that a twofold increase in RNA polymerase led to a twofold increase in total mRNA; but, total protein synthesis remained unchanged. An RNA polymerase underproducing system was also constructed; when RNA polymerase levels were reduced to 50% of a suitable control, total mRNA synthesis was also halved; yet, total protein levels were invariant.[21]

Possible translational limitations to recombinant-gene expression were investigated in our lab with a copy-number-mutant series of plasmids. Chemostat culture studies have shown cloned-gene expression reaches a plateau even as synthesis rates and steady-state levels of β-lactamase mRNA continue to increase.[27] It was found that although ribosomal RNA synthesis increases, steady-state cellular ribosomal RNA levels drop significantly as gene expression is increased by elevating copy number. This indicates one limitation to increased levels of cloned-gene production may be a deficiency in the translation machinery.[27]

To investigate further the nature of the limitations in recombinant protein production, it was decided to alter the transcription rate of a reference protein on a stably maintained plasmid and measure the impact of this mode of regulation of cloned-gene expression on mRNA synthesis and accumulation as well as on ribosomal RNA synthesis and steady-state levels. To facilitate this objective, plasmid pTKW106 was constructed.[26] This pUC derivative produces large quantities of β-galactosidase while achieving 100% plasmid stability within the chemostat.[26] Transcription from the *tac* promoter is tightly-regulated since the plasmid contains a copy of the $lacI^Q$ repressor; multiple copies of the *tac*

* To whom all correspondence should be addressed.

Biotechnology and Bioengineering, Vol. 38, Pp. 397–412 (1991)

CCC 0006-3592/91/040397-16$04.00

promoter arising from multiple copies of the plasmid can titrate a chromosome-based *lacI* repressor.[25] With this system, transcription of *lacZ* can be induced over a wide range by adding the non-cleavable lactose analog IPTG to the fermentation medium. In batch fermentations, β-galactosidase activity has been varied over a 2×10^4-fold range (unpublished data). The *parB* locus preserves plasmid segregational stability by killing cells which lose the plasmid via a reduction in the transmembrane potential.[26]

The chemostat was used to create a steady-state growth environment in which cells are forced to grow at the same rate for each fermentation. The same host and plasmid are used in each fermentation; therefore, the only difference in the metabolism of each culture is the degree of transcription of the plasmid-borne, cloned gene and translation of the resulting message.

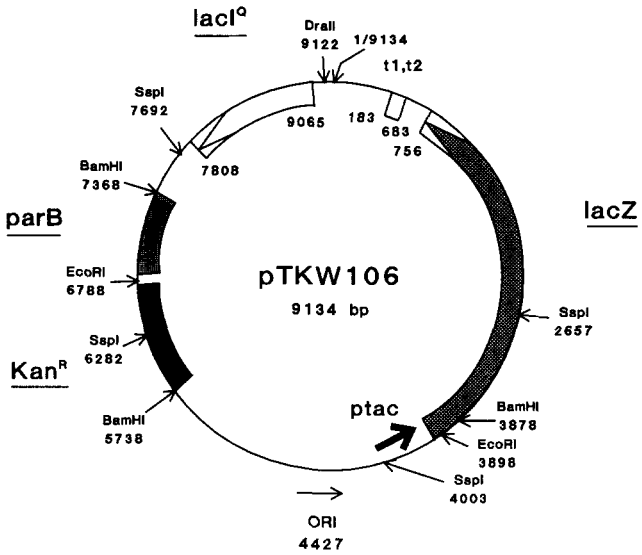

Figure 1. β-Galactosidase expression vector pTKW106.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *E. coli* strain BK6[26] was used as the host in the chemostat fermentations. Its genotype is *Δ(lacIPOZ)C29, lacY*$^+$*, hsdR, galU, galK, strA', leuB6, trpC9830, Δ(srl-recA)*306::Tn*10* with the result BK6 cannot produce β-galactosidase due to a genetically stable deletion while it retains the ability to produce β-galactoside permease. Hence, cells harboring plasmids which produce β-galactosidase form red colonies on MacConkey agar plates whereas plasmid-free cells form white colonies. Because of the *trp* mutation, it was necessary to supplement the minimal medium with tryptophan since casamino acids did not supply enough of this amino acid. The *recA* mutation was added to this host to increase plasmid stability by reducing homologous recombination.[26]

E. coli strain M182,[3] a parent strain of BK6, was used as the host for isolation of *lacZ* DNA from plasmid pMC1871. Its genotype is *Δ(lacIPOZYA)Z74, galU, galK, strA'*. The tightly regulated expression vector pTKW106[26] (9176 bp) is shown in Fig. 1. The plasmid includes the strong *tac* promotor upstream of the β-galactosidase gene, and the strong transcription terminators of the *rrnB* locus. For complete repression of the *tac* promoter, the *lacI*^{Q} gene has been included on the plasmid. The *parB* locus preserves plasmid stability by killing any host cells which lose the plasmid.[26] Kanamycin resistance is conferred by the *aphA* gene and is shown as Kan^R in Figure 1.

Hybridization Vectors and Plasmid DNA Manipulations

The source of the *lacZ* DNA used in the β-galactosidase mRNA-DNA hybridizations was plasmid pMC1871,[3] a pBR322 derivative. This 7478 bp vector contains the *lacZ* locus without the promoter, operator, ribosome binding site, and first eight amino-terminal amino acids.[3] The *lacZ* locus is surrounded by two multiple-cloning sites which allow the locus to be cleaved as a 3115 bp gene cartridge by cutting with the restriction enzyme *PstI*.

A large-scale plasmid prep was used to obtain pMC1871 plasmid DNA.[27] The protocol included overnight chloramphenicol amplification of plasmid DNA (in the presence of tetracycline selection pressure) followed by lysozyme-induced cell lysis, RNase treatment, precipitation of cellular debris with Triton surfactant solution, and PEG-induced DNA precipitation. The plasmid DNA was separated from chromosomal DNA using ultracentrifugation, and was restricted with *PstI*. The 3115- and 4363-bp fragments were separated by horizontal gel electrophoresis[15] using either a 0.6 or 1.2 wt % agarose gel and TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2.0 m*M* EDTA, 0.5 μg/mL EtBr, pH 8.5). The smaller band was cut from the gel and isolated as described previously.[27]

The 7500 bp fragment of plasmid pKK3535 containing the complete *rrnB* loci was used to quantitate the steady-state levels and synthesis rates of ribosomal RNA.[27] Isolation of this plasmid amd its *rrnB* locus has been described.[27]

Rapid plasmid isolations were conducted using the protocol of Rodriguez and Tait.[23] Briefly, 1.5 mL cell suspension was spun at 1.5×10^4 rpm in a microfuge, washed and resuspended in sucrose solution, then lysed with lysozyme in the presence of RNase. Sodium acetate was used to precipitate chromosomal DNA, and the plasmid DNA was precipitated from the supernatant using 2-propanol. The pDNA was washed with 80% ethanol and resuspended in TE buffer (10 m*M* Tris, pH 7.6, and 1 m*M* EDTA).

Media and Chemostat Cultivation

The fermentation medium used in the chemostat was M9 minimal medium[23] containing 0.2 wt % glucose, 0.4 wt % casamino acids, and 0.0018 wt % tryptophan. Isopropyl β-D-thiogalactoside (IPTG, US Biochemicals) was also added to the fermentation medium to induce *lacZ* (0.0–7.5 m*M*). Selection pressure was maintained for all of the chemostat fermentations by adding kanamycin at 100 μg/mL. Silicon antifoam was added at a concentration of 50 μg/mL. The medium was filter-sterilized with a 0.2-μm filter.

The fermentations were conducted at a dilution rate of 0.6 h^{-1} in a 3.0 liter Applikon ADI 1020 Bioprocessor/Fermentor.[27] The temperature was maintained at 37°C, the pH was controlled at 7.0, the agitation rate was fixed at 1000 rpm, the air flow rate was set at 122 mL/min, and the dissolved oxygen levels were monitored.

The number of viable cells at steady-state was determined for each chemostat culture by cell counts averaged over 10 MacConkey agar plates. The possibility of plasmid segregational instability was chekced by examining the MacConkey plates for white colonies. Structural stability of the plasmid was checked by horizontal electrophoresis.[15] No plasmid instability was indicated in any of the fermentations.

Total RNA

As described previously,[27] two samples of RNA (radiolabeled and unlabeled) were isolated under RNase-free conditions using the protocol of Dennis and Nomura.[5] Briefly, the isolation involved inducing cell lysis with SDS, extracting three times with redistilled phenol, and precipitating with ethanol. The RNA was centrifuged and resuspended in Tris/azide/EDTA buffer, and the phenol was removed by ether extractions. The DNA and protein were removed by DNase and pronase treatments. After additional phenol extractions and other ethanol precipitation, the RNA was resuspended in 2 × SSC buffer. The phenol was removed again by three ether extractions, and the resulting total RNA for each chemostat culture was quantified by averaging the spectrophotometrically-determined concentrations of the pulse-labeled and nonlabeled RNA samples.

β-Galactosidase Activity and Total Protein

The β-galactosidase activity was calculated based on the conversion of the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) to *o*-nitrophenol.[16] Ten millileters of each chemostat culture were thawed and centrifuged at 10^4 rpm for 5 min at 4°C. The cells were then resuspended in 0.75 mL of cold TEP buffer (100 m*M* Tris, pH 7.4; 10 m*M* EDTA, pH 7.0; 1 m*M* PMSF) and transferred to microcentrifuge tubes and placed on ice. The cells were then sonicated at 21 kH_z for one minute in two 30 s intervals using an Artek Dismembrator model 300 set at 30% and fitted with a microtip. A separate experiment in which cells were sonicated for up to two minutes revealed this sonication interval and intensity released the bulk of the β-galactosidase activity from the cells. Ten to twenty microliters of this cellular homogenate was then mixed with 2.5 mL reagent A (0.1 M Na_2HPO_4 adjusted to pH 7.3 with 0.1 *M* NaH_2PO_4), 100 μL reagent B (3.6*M* β-mercaptoethanol), 100 μL reagent C (30 m*M* $MgCl_2$), and 200 μL reagent D (33.2 m*M* ONPG in reagent A). The well-vortexed mixture was then placed in a Shimadzu UV160U Spectrophotometer with a constant temperature cell set at 25°C and wavelength fixed at 410 nm; the spectrophotometer then automatically calculated the change in absorbance over a two-minute interval. The activity of the β-galactosidase present, $\nu_{max,assay}$, was calculated using Beer's law as:

$$\nu_{max,assay} = \frac{\text{nmol ONPG cleaved}}{\text{min (mL assay)}} = \frac{\Delta C_{ONPG}}{t} = \frac{\Delta A}{\varepsilon l t} \quad (1)$$

where ΔC_{ONPG} is the change in ONPG concentration in the cuvette; t is time; ΔA is the change in absorbance; ε is the extinction coefficient for *o*-nitrophenol at 410 nm; and l is the light path length of the cuvette. Dilutions were then accounted for and the activity was converted to a cellular basis.

Sigma Protein Kit No. 690 was used to analyze the total protein content of cells of the chemostat culture. Both 50- and 100-μL samples of the sonicated cellular homogenate used for the β-galactosidase activity determinations were analyzed for each chemostat culture.

Synthesis Rates of Total RNA, β-Galactosidase mRNA, and rRNA

The synthesis rates of total RNA, β-galactosidase mRNA, and ribosomal RNA were found by pulse-labelling with uracil (^3H).[27] This included sampling the chemostat after steady-state was reached and pulse-labelling the cells for two minutes at 37°C and 250 rpm; the RNA was then isolated from the cells as described above.

The total RNA transcription rate was determined by adding 10 μL of the ^3H-RNA sample to 5 mL of Fisher Scinti Verse II liquid scintillation cocktail. The radioactivity of the sample was quantified using a Packard 1500 Tri-Carb Liquid Scintillation Analyzer.

The synthesis rate of β-galactosidase mRNA and rRNA were determined as follows. After binding the complementary DNA sequence (derived from a large-scale plasmid prep) to nitrocellulose using a slot-blot filtration unit, ^3H-RNA from each chemostat culture was then hybridized against the DNA dots for 60–70 h. Equal amounts of (μg) of RNA (isolated from the chemostat experiments) were suspended in a constant volume of buffer and used in the hybridizations. After

a series of stringent washes, the radioactivity of the hydrogen-bonded mRNA or rRNA on the nitrocellulose filter was quantified by the Packard liquid scintillation counter.

As reported before for the ribosomal RNA–DNA hybridizations,[27] an excess hybridization was performed to ensure that the bound *rrnB* DNA locus was always in excess of the rRNA transcripts contained within the total cellular RNA. It was found that the hybridizations should be executed such that the ratio should be less than (1.3 μg RNA)/(μg ribosomal DNA).[27] The hybridizations used in these experiments were designed to satisfy this requirement.

To determine the appropriate hybridization conditions for the β-galactosidase mRNA–DNA hybridizations, another excess hybridization experiment was performed. In this experiment, the amount of RNA which saturates the *lacZ* DNA was determined by binding 2.0 μg *lacZ* DNA to nitrocelluose filters. This DNA was obtained as a 3115-bp fragment of plasmid pMC1871. Pulse-labeled RNA isolated from the 1.0 m*M* IPTG chemostat culture was hybridized against this *lacZ* DNA and against salmon testes DNA (control) in amounts of 1, 2, 6, 12, and 24 μg. Duplicates were used for each level of RNA. The 1.0 m*M* IPTG chemostat culture was chosen because it has one of the highest levels of β-galactosidase mRNA per μg of RNA. As shown in Figure 2, the response remained linear up to 12.0 μg RNA/(2.0 μg DNA); therefore, a ratio of 6.0 μg RNA/μg DNA was used to ensure that the *lacZ* DNA probe was in excess of β-galactosidase mRNA. Above this level of RNA, the bound DNA becomes saturated with RNA, preventing an accurate measure of β-galactosidase mRNA levels within the cell.

Steady-State Levels of rRNA and β-Galactosidase mRNA

The steady-state levels of rRNA and β-galactosidase mRNA were found by hybridizing nitrocellulose dots with bound, nonradioactive, chemostat-derived, cellular RNA against an oligo-labeled DNA probe.[27] The radioactive DNA probe consisted of the locus specific for the transcript of interest and was isolated from plasmid DNA obtained from a large-scale plasmid prep. After hybridizing for 60–70 h at 42°C, the nitrocellulose dots were washed, and the radioactive signal from the hydrogen-bonded DNA was quantified using a liquid scintillation counter.

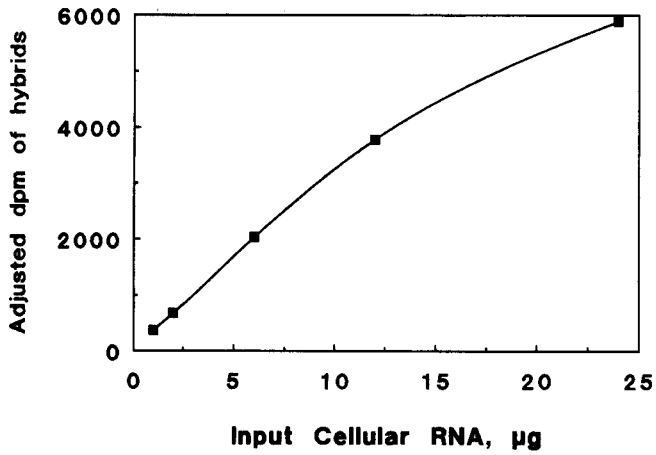

Figure 2. Hybridization to *lacZ* DNA, with line drawn to indicate trend.

Degradation Constants from Transcription Rate Equations

In order to quantify the loss of β-galactosidase mRNA in the cell due to degradation, the decay rate has been assumed to be proportional to the mRNA concentration.[27] Therefore, the β-galactosidase mRNA degradation rate term, $k_{d,\mathrm{mRNA}_{\beta\text{-gal}}}$, may be calculated by experimentally measuring the rate at which mRNA is synthesized ($r_{f,\mathrm{mRNA}_{\beta\text{-gal}}}$) and the steady-state concentration of this transcript ($[m\mathrm{RNA}]_{\beta\text{-gal}}$)[27]:

$$k_{d,\mathrm{mRNA}_{\beta\text{-gal}}} = \frac{r_{f,\mathrm{mRNA}_{\beta\text{-gal}}}}{[\mathrm{mRNA}]_{\beta\text{-gal}}} - \mu \qquad (2)$$

The specific growth rate, μ, is fixed by the dilution rate of the reactor system; hence, it is fixed by the experimenter. This monomolecular decay constant indicates the stability of mRNA and reveals the extent to which the degradation rate is affected by induction of transcription of the cloned gene.

Ribosomal RNA was analyzed using the same technique. The specific degradation rate for ribosomal RNA can be calculated as:

$$k_{d,\mathrm{rRNA}} = \frac{r_{f,\mathrm{rRNA}}}{[\mathrm{rRNA}]} - \mu \qquad (3)$$

SDS–PAGE

The individual protein bands of the chemostat cells were separated using SDS–PAGE with both stacking and separating gels and the discontinuous buffer system of Laemmli.[13] Cellular protein was isolated from 20 mL of thawed chemostat culture according to the method of Miller et al.[17] for each of the chemostat fermentations. Using the data of the total protein determinations and chemostat cell counts, a 3.5 wt % polyacrylamide stacking gel and a 12 wt % separating gel was then loaded such that each lane contained protein from 9.4×10^7 cells for each chemostat run (37–70 μg). Cellular protein isolated from the host BK6 and the high and low molecular weight standards of *Bio-Rad* (the higher-molecular-weight standard includes β-galactosidase) were run along with the chemostat samples. The gel was then electrophoresed and stained with coomassie blue. The fraction of cellular protein containing β-galactosidase

and RNA polymerase was determined by scanning densitometry using a Hoefer Scientific Instruments Scanning Densitometer (model GS 300) to scan the gel. The output was recorded using Fisher Recordall Series 5000 Chart Recorder.

Copy Number Determinations

The copy number of plasmid pTKW106 in the chemostat cultures was determined using horizontal agarose gel electrophoresis. Samples from each of the seven chemostat cultures were thawed, and the sample volume containing 9.5×10^8 cells for each culture was mixed with 1.0 mL of M182/pMC1871 reference cells. The reference cells contained a 7.5-kb plasmid (pMC1871) which provided an internal standard for monitoring the recovery of the chemostat plasmid DNA.[18] The cells were then spun at 10^4 rpm for 15 min at 4°C. A miniplasmid prep following the protocol of Rodriguez and Tait[23] was then performed. The chemostat and reference plasmids were then digested with *Eco*O109I restriction enzyme; control plasmids pMC1871 and pTKW106 (0.5 μg each) that had been isolated earlier using a CsCl-EtBr protocol were also digested with *Eco*O109I. The plasmids were then separated (in the presence of EtBr) using horizontal gel electrophoresis with a 0.6 wt % agarose gel.[15] With the gel illuminated by a Fotodyne 3-3000 Transilluminator, photographs were taken with a Polaroid MP-4 Land Camera using T665 film which produces both a negative and a positive print. To quantify the fluorescence intensity of each plasmid band, a Hoefer Scientific Instruments Scanning Densitometer (model GS 300) linked to a Fisher Recordall Series 5000 Chart Recorder was used to scan the negative.

RESULTS

The results of the chemostat experiments are presented below. In interpreting this data, it should be kept in mind that each chemostat experiment is a steady-state culture, and that each of the figures reveal a progression of steady-states in which the cells are stressed by the increasing metabolic load of producing the cloned-gene product (β-galactosidase). Hence, the data represent the steady-state response of the cell and not transient reactions.

β-Galactosidase Activity

The intracellular β-galactosidase activity plotted on a cellular basis is shown in Figure 3. Initially, from 0.0 to 1.0 m*M* IPTG, the enzyme activity increases in direct proportion to the extent transcription is induced with IPTG. For example, a 100-fold increase in IPTG from 0.01 to 1.0 m*M* resulted in a 102-fold increase in activity per cell. However, as the level of IPTG was increased from 1.0 to 7.5 m*M*, the cellular enzyme activity

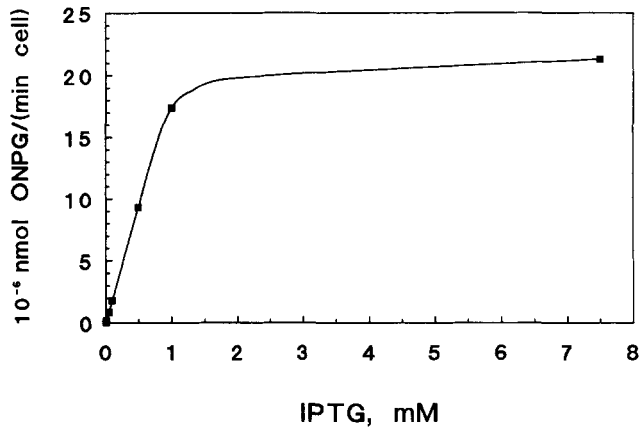

Figure 3. β-Galactosidase activity vs. IPTG, with line drawn to indicate trend.

reaches a maximum: the 7.5-fold increase in IPTG from 1.0 to 7.5 m*M* results in only a 23% increase in activity.

The relatively small increase in activity at high IPTG concentrations (1.0–7.5 m*M*) is probably due to nearly complete titration of the *lacI*^{Q} repressor protein by IPTG. As the IPTG concentration is increased beyond 1.0 m*M*, the lactose analog is less effective in increasing transcription since nearly all of the repressor molecules are already bound by IPTG. Hence, the *tac* promoter is nearly fully induced at 1.0 m*M* IPTG and additional IPTG does not significantly increase transcription of *lacZ*. It is also plausible that β-galactosidase protein production is becoming limited by the availability of ribosomes, and that degradation of the over-expressed protein may become enhanced.

The extracellular β-galactosidase activity was also measured and found to be negligible at all IPTG concentrations. The extracellular concentration averaged 1.4% for the seven chemostat fermentations and indicates that cell lysis was insignificant.

SDS-PAGE

The total cellular protein from 9.4×10^7 cells from each chemostat culture was separated on a polyacrylamide gel. As shown in Figure 4, the β-galactosidase (second arrow) produced per cell increases in intensity as expected as the level of IPTG is increased. The β-galactosidase band is first visible at 0.01 m*M* IPTG; at 1.0 m*M* IPTG the β-galactosidase band is 9% of the total protein produced by the bacterial cell as determined by scanning densitometry. The β-galactosidase band increases to 14 wt % of total cellular protein at 7.5 m*M* IPTG and is very distinct.

This level of activity in the chemostat corresponds well with the enzymatic activity found in batch cultures in which cells are grown for a time equivalent to the residence time of cells in the chemostat. The maximum β-galactosidase activity in batch cultures of BK6/pTKW106 is estimated to be 40–50 wt % (unpublished data).

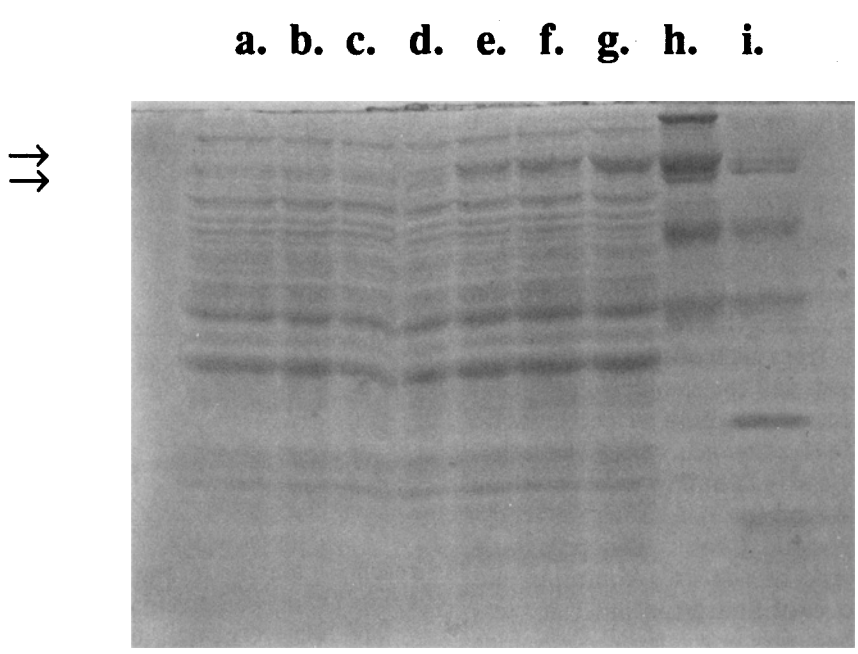

Figure 4. SDS–PAGE of total *E. coli* protein from chemostat cultures. Each gel well contains 9.4×10^7 cells of a chemostat culture. Lanes were loaded as follows: (a) 0.0 m*M*, (b) 0.01 m*M*, (c) 0.05 m*M*, (d) 0.1 m*M*, (e) 0.5 m*M*, (f) 1.0 m*M*, (g) 7.5 m*M* IPTG, (h) high-molecular-weight protein markers (myosin, β-galactosidase, phosphorylase B, BSA, ovalbumin) and (i) low-molecular-weight protein markers (phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme). Upper arrow indicates β and β′ subunits of RNA polymerase and lower arrow indicates the β-galactosidase band.

The RNA polymerase β and β′ bands are clearly visible on the SDS–PAGE gel (Fig. 4, top arrow). Their molecular weight is 145 and 155 kD,[10] respectively, and they migrate between the myosin (200 kD) and β-galactosidase (116 kD) molecular weight bands shown in lane h of Figure 4.

From the intensity of these two bands, the RNA polymerase concentration appears to remain constant as a function of total cellular protein for all seven of the chemostat fermentations. This is shown more clearly in Table I where the RNA polymerase concentration is listed for each chemostat fermentation as a fraction of total protein. Within the estimated error of 0.5%, the values are approximately constant. This indicates the cells continue to synthesize a constant amount (roughly) of this enzyme per microgram of protein at all levels of β-galactosidase expression.

Total Specific Transcription

By measuring the total radioactivity of the pulsed-labeled chemostat RNA samples, the total transcription rate (synthesis of *all* forms of RNA) was determined. On a cellular basis (dpm/min cell), the data are presented in Figure 9 and are discussed below with the ribosomal RNA synthesis rates. Total *specific* transcription rates (dpm/min μg RNA) may also be calculated by normalizing the measured radioactivity by the amount of RNA in each cell; hence the values reflect the *relative* amount of RNA that was newly synthesized as transcription increases from the *lacZ* locus. This data are plotted in Figure 5. The value for the chemostat experiment in which the plasmid-free host cells were grown in the absence of IPTG is labeled "BK6" in Figure 5.

The large increase (350%) in de novo RNA synthesis per microgram of RNA for the steady-state BK6/pTKW106 cells at 0.0 m*M* IPTG relative to the host BK6 indicates that the addition of the plasmid to the cells has a significant impact on cellular metabolism; the plasmid-bearing cells are forced to synthesize much

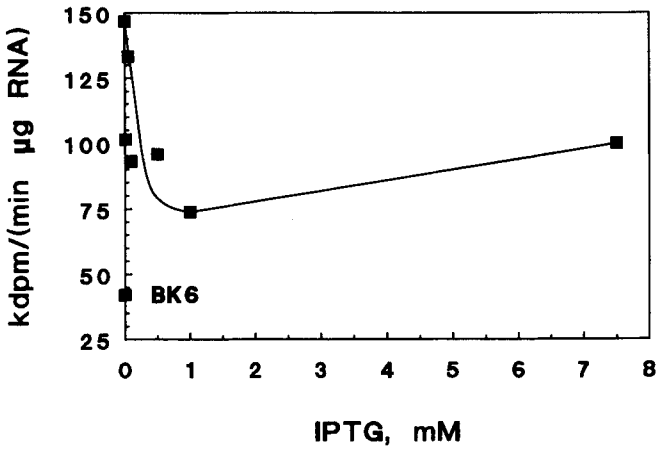

Figure 5. Total *specific* transcription vs. IPTG, with line drawn to indicate trend.

more RNA. This additional RNA consists of both rRNA and β-galactosidase mRNA since in the absence of the inducer IPTG, some β-galactosidase enzyme is made. The enzyme activity of the chemostat cells grown in the absence of IPTG, 0.079 nmol/(min μg protein), is 20-fold higher than the activity found in BK6/pTKW106 cells that are grown in batch cultures in the absence of IPTG.[28]

As the inducer concentration increases and the cell produces more β-galactosidase mRNA, there is a steady, twofold drop in specific transcription (Fig. 5). This indicates that the cells respond to further induction of the *lacZ* gene by synthesizing less RNA as a percentage of total RNA. The same trend was seen in five chemostat fermentations using host AMA1004 and plasmid pMJR1750. AMA1004 is a *recA*$^+$ version of BK6, and pMJR1750 is the precursor plasmid of pTKW106 which lacks the *parB* stability locus.[26] In this system, the specific transcription rate dropped 5.73-fold as the inducer concentration varied from 0.0 to 0.1 m*M* IPTG.

β-Galactosidase mRNA

Synthesis Rates of β-Galactosidase mRNA

Figure 6 presents the β-galactosidase mRNA synthesis rates as a function of induction of *lacZ* transcription with IPTG. The rate radioactive uracil is incorporated into the β-galactosidase mRNA of each cell (shown on the ordinate as dpm per minute per cell) is proportional to the rate β-galactosidase mRNA transcripts are synthesized on a cellular basis (transcripts per minute per cell).

As the level of inducer is increased from 0.01 to 7.5 m*M*, the synthesis rate increases steadily; overall there is 42-fold increase in transcription. The increase in *lacZ* transcription is nearly linear up to 1.0 m*M* IPTG; however, β-galactosidase mRNA synthesis does not increase linearly between 1.0 and 7.5 m*M* IPTG. This may be due to nearly complete titration of the *lacI*^{Q} repressor by IPTG.

The maximum of 288 × 10^{-6} dpm/(cell min) is comparable to the synthesis rate of rRNA (Fig. 9) at 7.5 m*M* IPTG, 385 × 10^{-6} dpm/(cell min), indicating approximately equal amounts (mass basis) of β-galactosidase mRNA and rRNA are being synthesized. This synthesis rate also agrees well with the 275 × 10^{-6} dpm/(cell min) reported previously[27] for β-lactamase mRNA synthesis in the highest copy number strain.

Steady-State Levels of β-Galactosidase mRNA

The steady-state cellular level of β-galactosidase mRNA is shown as a function of IPTG concentration in Figure 7. As the concentration of IPTG increases, the concentration of β-galactosidase mRNA rises, as expected, reflecting the increase in transcription shown previously in Figure 6. Overall, there is a 3.6-fold increase in the level of β-galactosidase mRNA. This increase is an order of magnitude less than that predicted by the rise in the synthesis rate when the cells are induced, indicating the rate of degradation of the cloned-gene transcript increases as the synthesis rate increases.

β-Galactosidase mRNA Degradation Constant

The β-galactosidase mRNA synthesis rates and steady-rate levels for each chemostat cultivation can be combined to determine the degradation constant $k_{d,\,\mathrm{mRNA}_{\beta\text{-gal}}}$ for this transcript. As shown in Eq. (3), $k_{d,\,\mathrm{mRNA}_{\beta\text{-gal}}}$ is calculated by dividing the synthesis rate by the steady-state level of the transcript and subtracting the specific growth rate μ. The proportionality constant between the two hybridizations was calculated as before.[27]

Figure 8 indicates $k_{d,\,\mathrm{mRNA}_{\beta\text{-gal}}}$ increases steadily as the cells are induced. Between 0.01 and 7.5 m*M* IPTG, the degradation constant increases 12-fold. This reveals that the decay mechanism is more active when the cell is highly stressed. Perhaps the excess β-galactosidase mRNA either triggers the synthesis of additional RNase II and polynucleotide phosphorylase or the existing pool of these two exoribonucleases becomes more

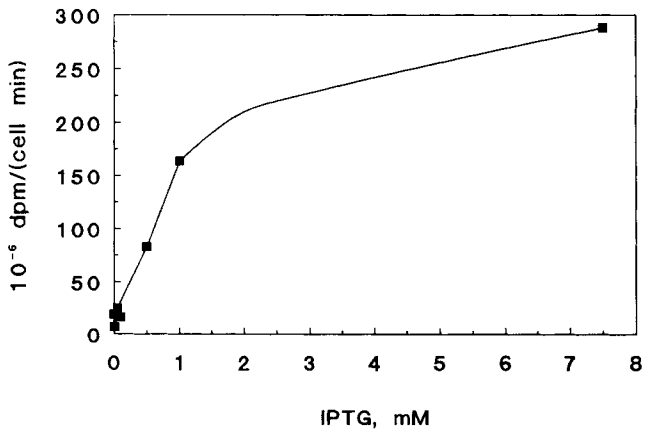

Figure 6. β-Galactosidase mRNA synthesis rates vs. IPTG, with line drawn to indicate trend.

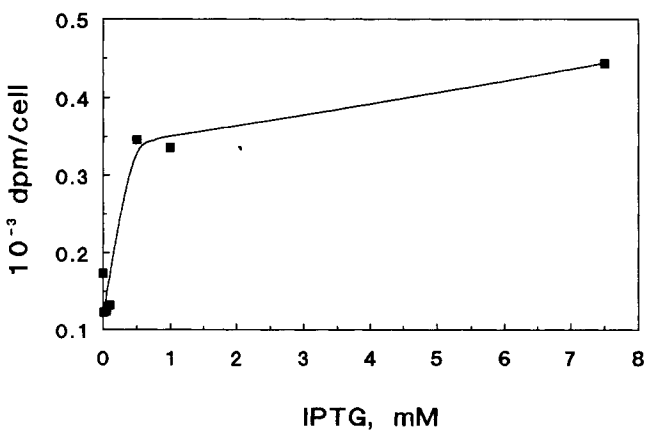

Figure 7. β-Galactosidase mRNA steady-state levels vs. IPTG, with line drawn to indicate trend.

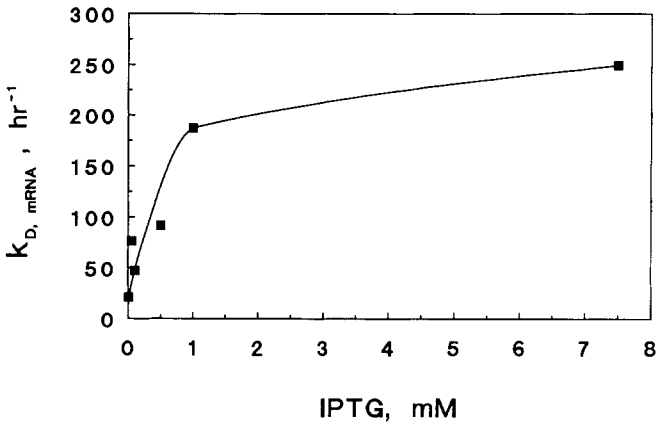

Figure 8. β-Galactosidase mRNA degradation rates vs. IPTG, with line drawn to indicate trend.

active.[19] As the *tac* promoter is induced and more mRNA is transcribed, abortive transcription may also occur. This may lead to the formation of incomplete transcripts that are rapidly degraded. Additionally, the β-galactosidase mRNA degradation constant may increase due to premature termination of translation.[20]

Ribosomal RNA

Synthesis Rate of rRNA

The effect of inducing β-galactosidase mRNA transcription on the synthesis rates of rRNA is presented in Figure 9. The rate rRNA is synthesized per cell is proportional to the rate uracil is incorporated into the rRNA transcripts; this is shown as dpm/(cell min) on the ordinate. Figure 9 also shows the synthesis rates of all forms of RNA as determined from measurements of total radioactivity.

The ribosomal RNA synthesis rate is maximum at 0.0 m*M* IPTG. It reaches a minimum at 0.1 m*M* IPTG after decreasing steadily 4.4-fold, then it increases 3.4-fold to roughly the initial synthesis level when the cells are fully induced at 1.0 and 7.5 m*M* IPTG.

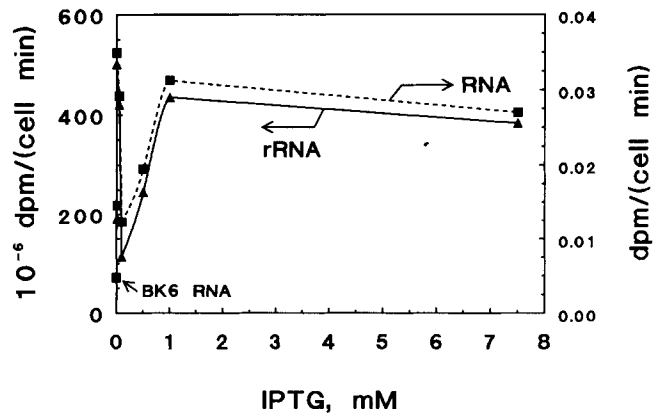

Figure 9. Total RNA and ribosomal RNA synthesis rates vs. IPTG, with lines drawn to indicate trends.

The total RNA synthesis rates follow a similar trend as *lacZ* in induced.

The high rRNA synthesis rate when the *lacZ* locus is not induced (0 m*M* IPTG) reflects the addition of the plasmid replication load to the cells and the expression of the constitutive plasmid genes. The high level of rRNA synthesis at 0 m*M* IPTG agrees well with the maximum that was measured for the total RNA synthesis (Fig. 9). Hence, the cell is able to produce the largest amount of rRNA at low levels of transcription induction and β-galactosidase enzyme production. As the metabolic load of producing β-galactosidase increases (0–0.1 m*M* IPTG), less priority is given to producing rRNA per microgram of total RNA (Fig. 5), and rRNA synthesis decreases. This reduction in rRNA synthesis may reflect the redirection of cellular energy away from rRNA synthesis and toward β-galactosidase mRNA synthesis.

The 3.8-fold increase in rRNA synthesis that begins at 0.1 m*M* IPTG corresponds to the 2.8-fold monotonic increase in rRNA synthesis seen when expression of β-lactamase is induced by amplifying copy number.[27] Beyond 0.1 m*M* IPTG, it appears as if a metabolic switch is activated that regulates rRNA synthesis. Perhaps the active ribosome population becomes depleted at high expression levels due to the increase in the number of ribosomes required to translate the ever increasing β-galactosidase mRNA concentration. Therefore, the cell is forced to replenish the ribosome pool and additional rRNA is synthesized. This increased synthesis of rRNA is reflected in the increase in the total RNA synthesis rate (Fig. 9) as expected.

Steady-State Levels of rRNA

As β-galactosidase production is induced with IPTG, the steady-state rRNA levels follow the trend shown in Figure 10. The dpm/cell signal indicated on the ordinate is proportional to the total number of rRNA transcripts in the cell. The number of rRNA transcripts should reflect the size of the ribosome population within the cell.

The steady-state rRNA curve mirrors that of the synthesis rates of rRNA (Fig. 9). After a 3.3-fold decrease in rRNA levels, a minimum is reached at 0.1 m*M* IPTG. The level of rRNA then increases 2.4-fold as the cells are further induced with IPTG levels beyond 0.1 m*M*. It appears that the size of the ribosome population is high initially, goes through a minimum, then increases to its original size as the *lacZ* gene is expressed.

Ribosomal RNA Degradation Constant

As with β-galactosidase mRNA, the ribosomal RNA synthesis rates and steady-state levels for each chemostat cultivation can be combined to determine the degradation rate $k_{d,rRNA}$ for this transcript. As shown in Eq. (4), $k_{d,rRNA}$ is calculated by dividing the synthesis rate by the

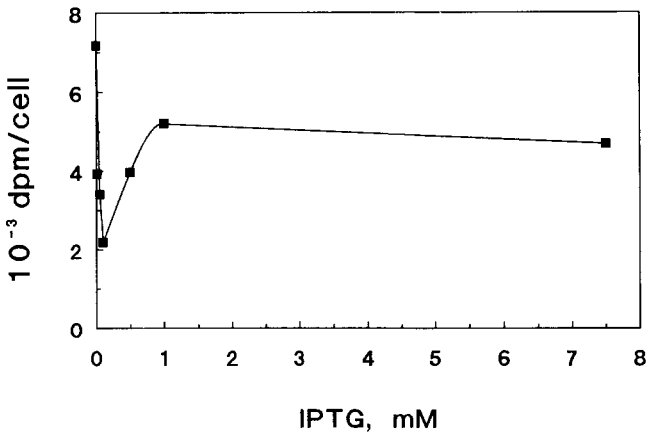

Figure 10. Ribosomal RNA steady-state levels vs. IPTG, with line drawn to indicate trend.

steady-state level of the transcript and subtracting the specific growth rate μ. The proportionality constant between the two kinds of hybridizations was calculated as described previously.[27]

The degradation rate data are presented in Figure 11. At 0.05 m*M* IPTG, where the rRNA synthesis rate is high (Fig. 9), the degradation rate obtains its highest value. At higher concentrations of IPTG, there is a general increase in the rate of degradation of the rRNA as β-galactosidase mRNA transcription is induced.

The rates calculated in these induction experiments are an order of magnitude lower than those involved when copy number is amplified.[27] This indicates that the ribosomal RNA is more stable in the induced-transcription system relative to the amplified-copy-number system.

Total RNA Levels

The effect of inducing transcription of β-galactosidase mRNA on the total RNA level of these bacterial cells is shown in Figure 12. Initially, the concentration of RNA per cell increases 230% beyond that of the plasmid-free cells revealing that the cell increases its RNA content due to the addition of the plasmid and expression of the cloned-gene. The cellular RNA content then decreases by 50% as the IPTG concentration increases from 0.0 to 0.1 m*M* IPTG. As the IPTG concentration rises to 1.0 m*M*, the RNA content increases 280% beyond the minimum level (0.1 m*M* IPTG).

This trend in RNA concentration was also seen in chemostat fermentations using the unstable plasmid system, AMA1004/pMJR1750. For this precursor system, the RNA concentration dropped 30% as the inducer level increased from 0.0 to 0.1 m*M*. The cellular RNA level then increased 40% as the IPTG level was increased to 0.5 m*M*. Hence, a minimum was consistently reached in the cellular RNA concentration at 0.1 m*M* IPTG. This minimum also agrees with those occurring in the rRNA synthesis and steady-state data and that of the total protein results. Apparently, the cell begins to synthesize more RNA once a certain level of induction of the cloned-gene is obtained.

Additionally, the maximum of 0.364 pg/cell of total RNA at 1. m*M* IPTG agrees well with the highest level of total RNA seen in the amplified-copy-number chemostat cultures (0.36 pg/cell). In that set of experiments, the host culture without a plasmid had the highest level of RNA. This value appears to be the maximum amount of RNA that the cell can produce while growing at this rate.

The threefold increase in RNA between 0.1 and 1.0 m*M* IPTG for BK6/pTKW106 is the result of steady-state increases in both rRNA *and* β-galactosidase mRNA. Because the steady-state ribosomal RNA levels shown in Figure 10 increase only to 65% of the maximum that occurs for this RNA at 0.0 m*M* IPTG, it appears the increase in total RNA is not purely the result of an increase in rRNA but reflects a substantial increase in the level of β-galactosidase mRNA (whole or partially degraded transcripts).

Using the total RNA and steady-state rRNA data, it is possible to estimate the steady-state mRNA content in the cell as a function of induction. These results are shown in Figure 13. At 0.0 m*M* IPTG, there are

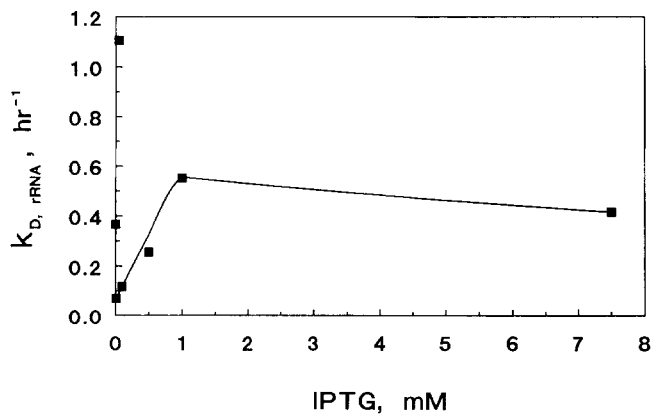

Figure 11. Ribosomal RNA degradation rates vs IPTG, with line drawn to indicate trend.

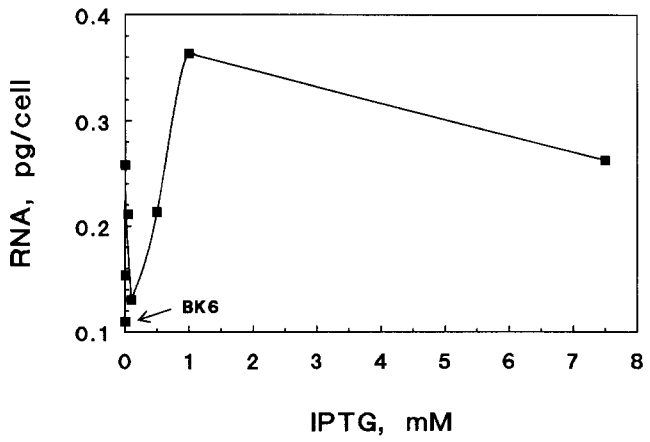

Figure 12. Total RNA vs. IPTG, with line drawn to indicated trend.

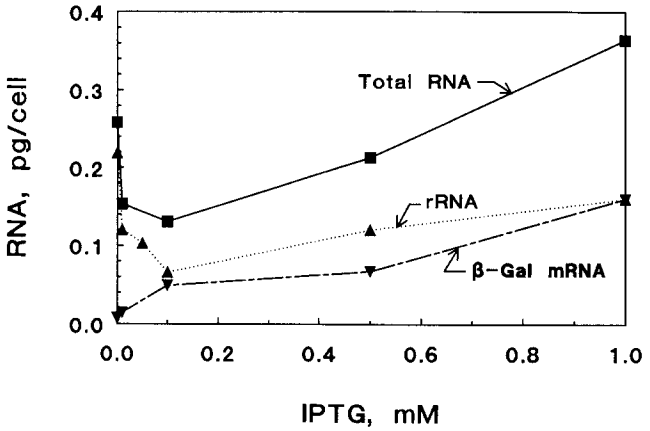

Figure 13. Total RNA, rRNA, and mRNA levels, with lines drawn to indicate trends.

0.258 pg RNA/cell (Fig. 12) of which 85% is rRNA[10] (assuming the *un-induced* recombinant cell has the same fraction of rRNA as a plasmid-free host). Furthermore, 12 wt % of the total RNA is tRNA,[10] a percentage that is assumed to not change with induction. Therefore, by multiplying the estimated amount of rRNA at 0.0 m*M* IPTG by the relative steady-state levels of rRNA shown in Figure 10, the rRNA levels are fixed; hence, the mRNA levels can be calculated as the remainder of total RNA less the tRNA fraction (12 wt %). Figure 13 reveals that at 1.0 m*M* IPTG, the cell must have only 0.16 pg rRNA and contains an equal amount of β-galactosidase mRNA. Since transcription of this mRNA increases 42-fold to synthesis levels on the order of rRNA production, a large increase in mRNA levels seems plausible.

Total Protein Levels

The total cellular protein levels are shown as transcription is induced with IPTG in Figure 14. As with the total RNA levels, the total protein concentration of the plasmid-free cells (BK6) is lower than that of the uninduced, plasmid-bearing cells (BK6/pTKW106 at 0 m*M* IPTG). The addition of the plasmid causes the protein level to increase by 200% whereas the total RNA levels increased 230%.

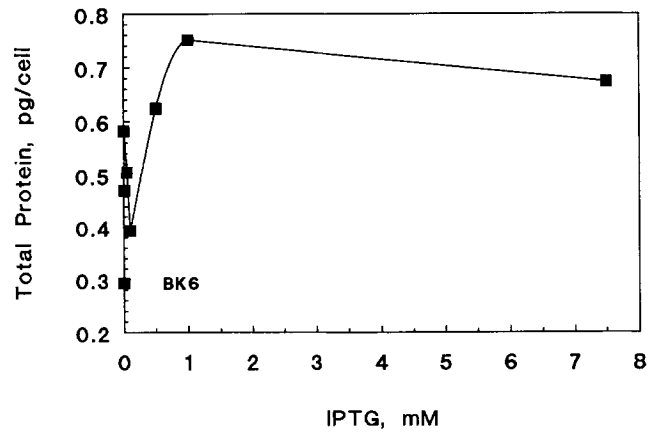

Figure 14. Total protein vs. IPTG, with line drawn to indicate trend.

As transcription is induced by increasing the IPTG concentration beyond 0.0 m*M*, the total protein levels on a cellular basis go through a minimum at 0.1 m*M* IPTG (Fig. 14). The protein content of the cells then increases by a factor of two to their highest level at 1.0 m*M* IPTG. This increase in total protein beyond the minimum probably reflects the increase in β-galactosidase protein seen in the SDS–PAGE gels.

Since both the total protein and total RNA concentrations are consistently higher for the plasmid-bearing chemostat cells than the plasmid-free hosts, it appears that one result of adding plasmids is to increase either the cell size or the concentrations of these two cellular components. The increase in the concentration of the RNA and protein may be account for the steady reduction in the specific growth rate that occurs upon induction of the *lacZ* locus with IPTG.[26]

Copy Number

To determine the structural stability and copy number of the plasmids, a 0.6 wt % agarose gel was run using the plasmid DNA isolated by a mini-prep from 9.5×10^8 cells taken from each chemostat culture. In order to overcome any errors in the rapid plasmid isolation protocol, the same amount of reference cells were added to each chemostat culture sample, so the ratio of the chemostat plasmid (pTKW106) to reference plasmid (pMC1871) should indicate the relative amount of plasmid DNA for each of the samples. Figure 15 shows the plasmids isolated by horizontal electrophoresis, and Table II indicates the ratios of the scanning densitometer signal of plasmid pTKW106 relative to the reference plasmid for each chemostat culture. Since the plasmid DNA ratios are roughly constant for each of the seven chemostat cultures (average of 2.24), the copy number of plasmid pTKW106 remained steady as β-galactosidase production was induced with IPTG in the chemostat.

This result is also obvious from the 0.0, 0.5, 1.0, and 7.5 m*M* IPTG chemostat samples shown in Figure 15. For these four samples, the reference plasmid bands are

Table I. Scanning densitometer results for RNA polymerase concentrations as a percentage of total protein.

Chemostat IPTG level (m*M*)	RNA polymerase concentration (% total protein)
0.00	1.3
0.01	1.7
0.05	1.9
0.10	1.0
0.50	1.6
1.00	1.5
7.50	1.3

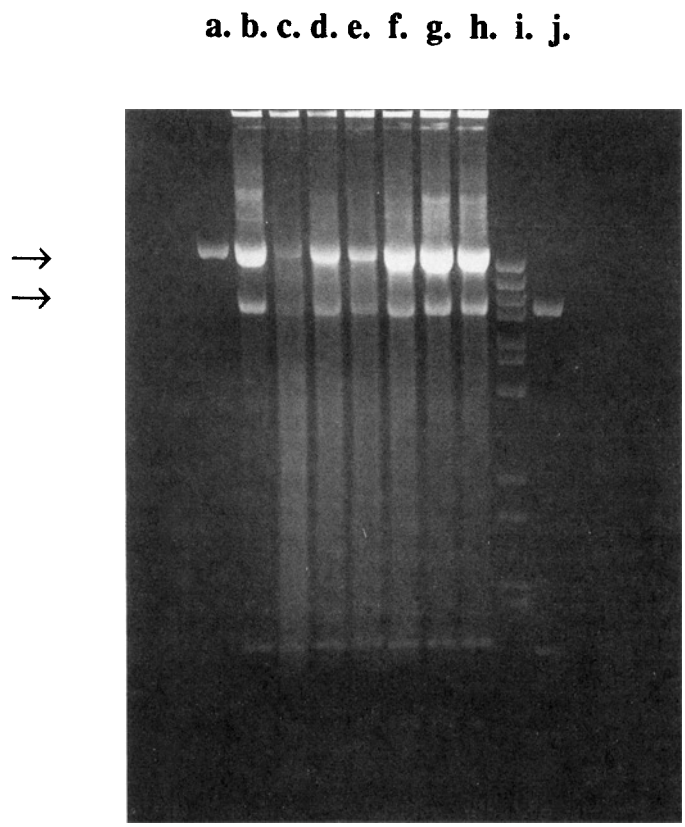

Figure 15. Copy number determination of plasmids from chemostat cultures. Upper arrow indicates pTKW106 and lower arrow indicates reference plasmid pMC1871. For each chemostat culture, plasmid DNA from 9.5×10^8 cells was isolated. The gel wells contain (a) control pTKW106 (500 ng), (b) chemostat culture sample 0.0 m*M*, (c) 0.01 m*M*, (d) 0.05 m*M*, (e) 0.1 m*M*, (f) 0.5 m*M*, (g) 1.0 m*M*, (h) 7.5 m*M* IPTG, (i) lambda DNA molecular weight markers (*Bst*E digest), and (j) control pMC1871 DNA (500 ng).

Table II. Scanning densitometer results for determining copy number.

Chemostat IPTG level (m*M*)	Ratio pTKW106 band intensity to reference plasmid band
0.00	2.4
0.01	2.2
0.05	2.0
0.10	1.9
0.50	2.4
1.00	2.4
7.50	2.3

the same intensity, so the chemostat plasmid bands can be directly compared; all four have the same intensity indicating constant copy number.

Also, each plasmid band migrated at the same rate as the control pTKW106 band indicating each had the same size. Hence, there was no structural change within the plasmids during the chemostat fermentations.

A comparison of the intensity of the pTKW106 plasmid band of the 0.0 m*M* IPTG chemostat culture with that of the intensity of the 500 ng control pTKW106 DNA added to the agarose gel revealed that the chemostat plasmid band has more DNA by a factor of 2.36, assuming the fluorescence of EtBr is proportional to the amount of DNA loaded on the gel. Hence, an estimate of the copy number of pTKW106 can be made since both the size of the plasmid and number of cells from which the plasmid DNA was derived are known. Based on three gels, the copy number appears to be about 84 for these cells in minimal media supplemented with casamino acids. This result seems reasonable since pTKW106 is derived from pUC18 which is a high copy number derivative of pBR322. It has been determined that the pUC plasmid series has a copy number of approximately 130,[22] and plasmid pBR322 has a copy number of about 22 under these growth conditions.[14]

DISCUSSION

Plasmid Stability and Copy Number

The expression vector pTKW106 was 100% stable in host BK6 in the seven chemostat experiments in which expression of β-galactosidase was induced with IPTG. No plasmid-free segregants were generated, and the

plasmids from the chemostat cultures all migrated with the control pTKW106 plasmid band (as shown by agarose gel electrophoresis in Fig. 15). Furthermore, for each chemostat fermentation, the ratio of intensity of the plasmid band to the reference plasmid band was constant. Therefore, the vector was both segregationally and structurally stable and was maintained at a constant 84 copies per chromosome equivalent.

Hence, the experimental data shown in the previous section are derived from a homogeneous population of cells at steady-state which consistently harbor a 9176-bp plasmid at constant copy number. Any differences in cell metabolism between the cultures are the result of different levels of cloned-gene transcription and expression and are not the result of differences in replication of the plasmid. It is tacitly assumed that the other plasmid-borne proteins, the constitutively expressed *lacI*^{Q} and *aphA*(Kan^{R}) proteins, are produced at the same level as IPTG concentrations vary.

β-Galactosidase Activity

The plateau seen in the β-galactosidase activity at high expression levels is similar to that seen in the β-lactamase activity when copy number is elevated.[27] Both systems indicate that recombinant protein production increases linearly as transcription or copy number are initially increased; yet, protein production reaches a limitation at high expression levels. For the IPTG-induced cultures, the plateau occurs at relatively high levels of IPTG (1.0–7.5 m*M*) because the *lacI*^{Q} repressor is present on the plasmid itself; hence, there are 84 copies of the repressor rather than the single copy that would be found if the repressor were on the chromosome.

In contrast to the amplified-copy-number system[27] where each plasmid adds an additional promoter site, the β-galactosidase plateau seen in the IPTG-inducible strain may be due to both limitations in the translational machinery as well as nearly complete titration of *lacI*^{Q} repressor. Apparently two regimes are present for transcription induction. At IPTG concentrations below 1.0 m*M*, additional inducer molecules lead to a stoichiometric increase in transcription induction as shown in Figures 3 and 6. Above 1.0 m*M* IPTG, almost all of the *lacZ* promoters are fully induced (due to nearly complete association of the repressor molecules with IPTG) and additional IPTG does not lead to a linear increase in *lacZ* transcription.

It should be emphasized that active enzyme levels have been assayed (activity) rather than measuring the broader indicator of protein production which includes the total pool of β-galactosidase enzyme. This pool includes both active and inactive (denatured) protein and degraded polypeptides derived from β-galactosidase. Some degradation of the β-galactosidase enzyme probably occurs as it is likely that proteolytic enzymes are activated at high expression levels. This premise could be checked by a western blot where degraded forms of β-galactosidase could be investigated as a function of induced expression.

Protein denaturation is indeed implicated when the SDS–PAGE results are compared to the enzyme activity data. From the SDS–PAGE gel (Fig. 4), it is clear the level of β-galactosidase protein continues to increase as a fraction of cellular protein as induction rises. From 1.0 to 7.5 m*M* IPTG, this level increases by approximately 60%; however, there is only a 22% increase in β-galactosidase cellular activity over the same induction range. It appears that 63% of the additional β-galactosidase that is produced by the cell at 7.5 m*M* IPTG is rendered inactive (denatured) or all the enzyme has reduced specific activity. This additional β-galactosidase does not include proteolytic degradation since this form of the enzyme migrates at 116 kD.

β-Galactosidase mRNA Synthesis and Degradation

As transcription of β-galactosidase mRNA is induced, the large, linear increase in β-galactosidase activity (460-fold) and the increase in the β-galactosidase protein band seen on the SDS–PAGE gel both indicate that recombinant protein production is linked, to a large extent, to the rate of transcription of the cloned gene. However, the hybridization experiments indicate the steady-state level of β-galactosidase mRNA is not altered greatly. This seems to indicate that each transcript is quickly degraded and that the stability of the transcript is important for β-galactosidase production.

Using the measured synthesis rates and steady-state levels of β-galactosidase mRNA (Figs. 6 and 7), it was determined that the transcript is degraded more rapidly as the rate of transcription increases (Fig. 8). This increase in the degradation rate is reflected in the 12-fold increase in the specific degradation rate constant for β-galactosidase mRNA. This increase agrees well with the 13.5-fold increase in the β-lactamase degradation constant that was measured at high expression for the copy-number mutant plasmid system.[27] Apparently, mRNA degradation becomes more important as expression of the recombinant protein increases in both the elevated copy-number (β-lactamase) and chemically inducible (β-galactosidase) systems.

Since the synthesis rate of β-galactosidase mRNA increases steadily with increasing transcription induction, protein production is probably not limited by the capability of the cell to *synthesize* mRNA. Even at high expression levels (from 1.0 to 7.5 m*M* IPTG), β-galactosidase mRNA synthesis increases 75%, resulting in a 32% increase in the steady-state levels of this transcript and a 23% increase in the enzyme activity. In fact, when transcription is fully induced, the rate of β-galactosidase mRNA synthesis approaches the rate of ribosomal synthesis. Furthermore, the SDS–PAGE results indicate the level of RNA polymerase β and β′ subunits remains constant as a function of total pro-

tein throughout the range of induction. This indicates a shortage for this enzyme does not exist.

The same conclusion was drawn from the steady increase in both the synthesis rate and steady-state levels of β-lactamase mRNA in the amplifable-copy-number expression system.[27] The results of the chemically-inducible system, like those of the copy-number system, extend the results of Nomura who showed, for non-recombinant systems, that mRNA is synthesized in excess in *E. coli*.[21]

Even though β-galactosidase mRNA transcription continues to increase with increasing levels of IPTG, the *steady-state levels* of β-galactosidase mRNA increase only fourfold over the whole range of induction, an order of magnitude less than the increase in synthesis. In the copy-number system, it was estimated that the steady-state level increased 16-fold as copy number increased. Therefore, the stability of β-galactosidase mRNA probably does impact the total amount of recombinant protein produced since its half-life is only 1.5 minute,[7] half that of β-lactamase mRNA. It would be interesting to increase the stability of the β-galactosidase message by fusing a more stable RNA to it[4,7] and see the impact this has on recombinant protein production under the same induction conditions.

Ribosomal RNA Synthesis and Degradation

After going through a minimum at 0.1 m*M* IPTG, the ribosomal RNA synthesis rates increase 3.8-fold with increasing cloned-gene expression. This increase parallels the 2.8-fold monotonic increase in rRNA synthesis rates for the copy-number-mutant system. Both of these results agree well with the results of Gausing who showed *E. coli* can increase its rRNA transcription at least by a factor of 2.5 to 4.0 beyond that required for exponential growth rate in these chemostat experiments.[27] For both systems, the increase in rRNA production seems to indicate production of large amounts of recombinant protein requires the cell to synthesize additional ribosomes. The existing ribosome pool is depleted by increased translation of the recombinant message, and the cell responds by increasing rRNA synthesis in order to produce additional ribosomes.

However, for the transcription induction system, the steady-state concentrations of rRNA do not simply decrease as in the elevated copy-number system[27]; instead, the steady-state level of rRNA goes through a minimum at 0.1 m*M* IPTG and increases 2.4-fold at high induction levels. Since expression of the cloned gene is higher in the chemically induced system (14 wt % vs. ~ 2 wt % total cell protein), it appears rRNA regulation is pushed beyond the simple decrease in the steady-state rRNA levels seen in the amplified copy number system.

Additionally, the rRNA in the induction system is much more stable than that produced in the copy-number-mutant system. This increase in rRNA stability is reflected in its lower degradation constant (1/10 that of the copy-number mutant system). It appears that ribosomal protein production can keep pace with that of the rRNA so that functional ribosomes are produced from the additional rRNA synthesized. Hence, the newly synthesized RNA is stable and not degraded as it was in the copy-number mutant system. This result suggests that there is additional capacity for recombinant protein production in systems which induce transcription compared to systems which amplify copy number.

Although the ribosomal RNA levels do not steadily decrease (as in the copy-number mutant system) they are very important to the cell. This close relationship is shown by the trend in cellular protein production: it mirrors exactly the trend shown in the steady-state level of rRNA (cf. Figs. 10 and 14). Additionally, high recombinant protein production coincides with high rRNA steady-state levels.

Total RNA Synthesis and Steady-State Levels

The largest RNA synthesis capacity of the cell occurs as the first increment of β-galactosidase enzyme is made and the plasmid is added to the host as shown in Figure 5. This in consistent in that is has been shown that plasmid replication has a significant effect on cell metabolism; increasing the copy number or the size of the plasmid causes the maximum specific growth rate of the cell to decrease in minimal medium.[1,24] Since the growth rate is held constant in these chemostat experiments, the cell cannot reduce its growth rate. Instead, the cell responds by increasing rRNA synthesis to maintain its growth rate with the additional metabolic burden of the plasmid. This is reasonable since ribosome synthesis is closely related to growth rate: synthesis of ribosomes varies as the square of the specific growth rate for cells doubling at these rates.[11] In effect, replicating the plasmid and expressing its genes requires additional ribosomes; hence, the cell responds by increasing rRNA synthesis to maintain the population of ribosomes required for a specific growth rate of 0.6 h^{-1} as well as supply the ribosomes associated with the plasmid.

As the inducer concentration increases from 0.0 to 0.1 m*M*, it appears the increasing metabolic load of producing the cloned-gene product causes the cell to respond by producing less RNA (Fig. 5). However, all levels of RNA synthesis in the plasmid-bearing cells are at least 175% higher than that of the plasmid-free cells indicating that the cell redirects its cell machinery toward making RNA when cloned-gene products are produced.

The total RNA concentrations reflect the metabolic changes that occur in the cell as the cloned gene is expressed. Upon addition of the plasmid, the total RNA concentrations rise. The cellular RNA concentration is reduced at intermediate expression levels; but, increase again during high expression of *lacZ*. Since the total RNA consists chiefly of rRNA (up to 0.1 m*M* IPTG), these results substantiate the downward trend of the

steady-state rRNA data up to 0.1 m*M* IPTG. Beyond 0.1 m*M*, the net increase in total RNA reflects the increase in both rRNA and β-galactosidase mRNA.

Ribosomal RNA Synthesis Model

These results suggest a model for ribosomal RNA synthesis in bacterial cells in which a cloned gene is expressed by inducing a strong promoter. In effect, it appears that rRNA synthesis is regulated by the concentration of the nontranslating ribosome population, by the distribution of the RNA polymerase population, and by relaxation of attenuation of RNA polymerase along the rRNA transcript.

Upon addition of the plasmid to the host, the cell responds by increasing rRNA synthesis because of the additional translational requirements associated with plasmid replication and because of the net increase in transcripts that are generated as a result of the plasmid-borne genes. This additional mRNA requires additional ribosomes for translation. The increased translational load results from constitutive expression from each of the 84 copies of the plasmid (per chromosome) of both the Kan^R gene and $lacI^Q$ repressor gene as well as expression of *lacZ* (production of β-galactosidase enzyme is nonzero even at 0.0 m*M* IPTG). Due to this net increase in demand for ribosomes, the cell must produce more rRNA to prevent depletion of the active ribosome population.

This growth-associated regulation of rRNA synthesis appears to be responsible for the increase in rRNA synthesis that occurs when the plasmid is added to the host cell. It has been shown that free, nontranslating ribosomes (*assembled* rRNA and r-protein) are in equilibrium with active, translating ribosomes (polysomes), and that the nontranslating pool of ribosomes regulate rRNA synthesis at the level of transcription.[8,9,11] As the translational requirements of the cell increase, the nontranslating ribosome pool decreases. This reduction in the nonactive ribosome pool initiates rRNA synthesis either by enhancing rRNA promoter strength or RNA polymerase binding using the free ribosomes themselves or an effector molecule.[8]

The increase in rRNA synthesis that occurs upon addition of the plasmid is shown clearly in Figure 5; the total specific RNA synthesis increases dramatically from the host cells alone (BK6) to the plasmid bearing cells (BK6/pTKW106) at 0.0 m*M* IPTG. On a total RNA synthesis per cell basis (total dpm/min cell), the increase is 7.5-fold (Fig. 9). Since plasmid-based transcription is relatively low at 0.0 m*M* IPTG, the fraction of total RNA synthesis which is rRNA synthesis may be estimated as 50% at this growth rate.[6] Therefore, the large increase in total RNA synthesis represents a significant increase in the production of ribosomal RNA.

The rate of rRNA synthesis also depends on the fraction of RNA polymerase that is active and the fraction that is engaged at rRNA promoters.[2] Since the total protein levels decrease by 32% from 0.0 to 0.1 m*M* IPTG (Fig. 14), and with the concentration of RNA polymerase constant as a fraction of total protein at all levels of transcription induction (SDS–PAGE results, Table I), the total RNA polymerase concentration decreases in the cell. Additionally, β-galactosidase mRNA transcription is also induced; therefore, the number of active RNA polymerase molecules per total mRNA decreases due to the reduction in total RNA polymerase and the increase in the number of transcripts in the cell.

The rRNA loci on the chromosome also compete with the *lacZ* gene on each plasmid for RNA polymerase. It appears that as the *lacZ* promoter (*tac*) is derepressed by the addition of IPTG from 0.0 to 0.1 m*M*, the *tac* promoter titrates the RNA polymerase with the result that there is less transcription at the rRNA operons and rRNA synthesis decreases as shown in Figure 9. This titration of RNA polymerase by the plasmids seems reasonable since there are 12-fold more *tac* promoters than rRNA promoters (plasmid copy number 84 vs. 7 rRNA loci per chromosome[10]). The net effect is that the rRNA promoter strength is the same as upon addition of the plasmid; however, there is less RNA polymerase per rRNA transcript so rRNA synthesis decreases. Bremer et al. have also reasoned that partitioning of RNA polymerase between rRNA promoters and mRNA promoters controls synthesis of rRNA.[2]

A different mode of regulation of rRNA synthesis appears to become dominant for induction of the *tac* promoter beyond 0.1 m*M* IPTG since rRNA synthesis increases 2.8-fold from 0.1 to 1.0 m*M* IPTG (Fig. 9). Although the RNA polymerase population remains constant as a fraction of total cellular protein and total cellular protein increases 70% (Fig. 14), the increase in transcription is not solely the result of a slightly larger RNA polymerase concentration. As shown in Figure 6, there is a large increase (17-fold) in β-galactosidase mRNA which should more than offset the increase in the RNA polymerase concentration. Instead, the increased rRNA transcription may be the result of relaxation of transcription attenuation of existing RNA polymerase molecules on the rRNA loci. Apparently, increasing *lacZ* transcription continues to titrate RNA polymerase as before; however, a threshold is reached where rRNA synthesis is actually induced by the reduction in RNA polymerase molecules available for rRNA transcription.

It has been shown that the leader region of *rrnB* contains potential regulatory sequences which act at the level of RNA chain elongation (transcription attenuation and antitermination) and promoter strength.[8,12] Specifically, in vitro results have demonstrated that a transcription termination site exists 260 bp downstream from the P1 promoter, and 8 transcription pause sites exist 47, 63, 77, 78, 90, 91, 119, and 126 bp downstream from the P1 promoter.[12] Transcription termination and attenuation at these sites are dependent on concentrations of the regulatory nucleotide guanosine tetra-

phosphate (ppGpp) as well as the NusA and NusB proteins.[8,12] Additionally, binding of RNA polymerase to the promoters of *rrnE* and *rrnB* rRNA loci is also decreased by the addition of ppGpp so *both* a reduction in promoter strength *and* transcription attenuation can reduce rRNA synthesis.[12]

Relaxation of transcription attenuation at these sites could potentially increase the synthesis rate of rRNA six-fold[12] so it is a very effective mode of regulation. Hence, if this transcription attenuation is relaxed at the rRNA loci as transcription of the *lacZ* gene increases, rRNA synthesis would be expected to increase significantly.

This mode of regulation seems reasonable in that the extent to which RNA polymerase pauses at the 90 and 91 bp sites of *rrnB* has been shown to decrease as the concentration of RNA polymerase decreases.[12] At high RNA polymerase concentrations, the pausing of the enzyme at this site blocks transcription initiation from both the P1 and P2 promoters, and it has been reasoned that high RNA polymerase concentrations could abolish expression of *rrnB*.[12] As transcription from *lacZ* increases, the concentration of free RNA polymerase should decrease steadily and attenuation of RNA polymerase at the 90 and 91 bp sites should be reduced with the result that rRNA synthesis would again increase steadily.

This reduction in rRNA transcription attenuation due to shifting of the RNA polymerase population to the cloned gene (*lacZ*) agrees well with the experimental results since the bulk of the *lacZ* transcription occurs beyond 0.1 m*M* IPTG (Fig. 6). Hence, relaxation of transcription attenuation could explain the increase in rRNA synthesis that occurs as transcription of *lacZ* is induced beyond 0.1 m*M* IPTG.

This model also seems consistent with the rRNA synthesis results of the amplified-copy-number chemostat experiments. In these fermentations, rRNA synthesis increased steadily as the copy number increased from 0 to over 400.[27] Since the plasmid replication burden increases substantially (equal to 90% of chromosome replication at copy number 408), the model predicts the nontranslating pool of ribosomes should decrease as more ribosomes are required for translation of the proteins associated with replication and DNA precursor formation. Additional ribosomes are also required for translation of the plasmid-borne, cloned-gene message. Hence, the cell is forced to increase its rRNA synthesis in order to maintain this enhanced protein production capacity.

CONCLUSIONS

Strain BK6/pTKW106 is a stable expression system useful for studying chemically-induced, clone-gene production in *E. coli*. Using β-galactosidase as a reference protein, cloned-gene expression in bacterial cells does not appear limited by the synthesis of cloned-gene mRNA when a strong promoter is chemically induced. However, the stability of this transcript appears to play an important role at high levels of expression since the degradation rate of the β-galactosidase mRNA has been shown to increase with increasing induction.

By measuring rRNA synthesis rates and steady-state concentrations, it also has been shown that as the *lacZ* gene is expressed, rRNA synthesis rates and steady-state concentrations as well as total RNA concentrations go through a minimum. Therefore it appears the cell regulates its metabolism to increase rRNA synthesis upon addition of the plasmid to the host cell and upon high-level expression of the cloned-gene product.

These results imply limitations exist within the translational machinery of the cell since the largest level of RNA synthesis (primarily rRNA) occurs upon addition of the plasmid to the host cell when cloned-gene expression is at its lowest level. Since the total cellular protein levels mirror the ribosomal RNA concentrations at all levels of expression, the translational capacity of the cell remains a crucial factor in cloned-gene production.

This work was supported in part by the North Carolina Biotechnology Center (Grant 86-G-00722) and by a stipend from the Southeastern Regional Fellowship Program (for TKW). The authors would like to thank Dr. H. Noller for donating plasmid pKK3535 and R. Kuhn for donating M182/pMC1871. We are also grateful for the helpful comments of R. Kuhn and S. Thomas.

References

1. Betenbaugh, M. J., Beaty, C., Dhurjati, P. 1989. Effects of Plasmid Amplification and Recombinant Gene Expression on the Growth Kinetics of Recombinant *E. coli*. Biotechnol. Bioeng. **33**: 1425.
2. Bremer, H., Baracchini, E., Little, R., Ryals, J. 1988. Control of RNA Synthesis in Bacteria. In: Tuite, M. F., Picard, M., Bolotin-Fukuhara, M. (ed.), Genetics of Translation: New Approaches. Springer-Verlag, New York.
3. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K., Chou, J. 1983. β-Galactosidase Gene Fusions for Analyzing Gene Expression in *Escherichia coli* and Yeast. Methods Enzymol. **100**: 293.
4. Chan, W. K.Y., Belfort, G., Belfort, M. 1988. Stability of Group I Intron in *Escherichia coli* and Its Potential Application in a Novel Expression Vector, Gene. **73**: 295.
5. Dennis, P. P., Nomura, M. 1975. Regulation of the Expression of Ribosomal Protein Genes in *Escherichia coli,* J. Mol. Biol. **97**: 61.
6. Gausing, K. 1977. Regulation of Ribosome Production in *Escherichia coli:* Synthesis and Stability of Ribosomal RNA and of Ribosomal Protein Messenger RNA at Different Growth Rates. J. Mol. Biol. **115**: 335.
7. Gorski, K., Roch, J. M., Prentki, P., Krisch, H. M. 1985. The Stability of Bacteriophage T4 Gene 32 mRNA: A 5' Leader Sequence That Can Stabilize mRNA Transcripts. Cell. **43**: 461.
8. Gourse, R. L., Sharrock, R. A., Nomura, M. 1985. Control of Ribosomes Synthesis in *Escherichia coli*. In: Hardesty, B., Kramer, G. (ed.), Structure, Function, and Genetics of Ribosomes, Springer-Verlag. New York.
9. Gourse, R. L., Takebe, Y., Sharrock, R. A., Nomura, M. 1985. Feedback Regulation of rRNA and tRNA Synthesis and Accumulation of Free Ribosomes After Conditional Expression of rRNA Genes. Proc. Nat. Acad. Sci. **82**: 1069.

10. Ingraham, J. L., Maaloe, O., Neidhardt, F. C. 1983. Growth of the Bacterial Cells. Sinauer Associates, Sunderland, MA.
11. Jinks-Robertson, S., Gourse, R. L., Nomura, M. 1983. Expression of rRNA and tRNA Genes in *Escherichia coli:* Evidence for Feedback Regulation by Products of rRNA Operons. Cell **33**: 865.
12. Kingston, R. E., Chamberlin, M. J. 1981. Pausing and Attenuation of in Vitro Transcription in the *rrnB* Operon of *E. coli.* Cell. **27**: 523.
13. Laemmli, U. K. 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, Nature. **227**: 680.
14. Lin-Chao, S., Bremer, H. 1986. Effect of the Bacterial Growth Rate on Replication Control of Plasmid pBR322 in *Escherichia coli.* Mol. Gen. Genet. **203**: 143.
15. Maniatis, T., Fritsch, E. F., Sambrook, J. 1982. In: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. Miller, D. M. lecture notes, Zoology 480, North Carolina State University.
17. Miller, D. M., Stockdale, F. E., Karn, J. 1986. Immunological Identification of the Genes Encoding the Four Myosin Heavy Chain Isoforms of *Caenorhabditis elegans.* Proc. Natl. Acad. Sci. USA. **83**: 2305.
18. Moser, D. R., Campbell, J. L. 1983. Characterization and Complementation of pMB1 Copy Number Mutant: Effect of RNA I Gene Dosage on Plasmid Copy Number and Incompatibility. J. Bacteriol. **154**: 809.
19. Neidhardt, Frederick C. 1987. *Escherichia coli* and *Salmonella Typhimurium:* Cellular and Molecular Biology, American Society for Microbiology, Washington, D.C.
20. Nilsson, G., Belasco, J. G., Cohen, S. N., von Gabain, A. 1987. Effect of Premature Termination of Translation on mRNA Stability Depends on the Site of Ribosome Release. Proc. Nat. Acad. Sci. **84**: 4890.
21. Nomura, M., Bedwell, D. M., Yamagishi, M., Cole., J. R., Kolb, J. M. 1987. RNA Polymerase and Regulation of RNA Synthesis in *Escherichia coli:* RNA Polymerase Concentration, Stringent Control, and Ribosome Feedback Regulation. In: Reznikoff, W. S. (ed.), RNA Polymerase and the Regulation of Transcription. Elsevier, New York.
22. Rodriguez, R. L., Denhardt, D.T. 1988. Vectors: A Survey of Molecular cloning Vectors and Their Uses. Butterworths, Boston.
23. Rodriguez, R. L., Tait, R. C. 1983. In: Recombinant DNA Techniques. An Introduction. Benjamin/Cummings Publishing Co., Menlo Park, CA.
24. Ryan, W., Parulekar, S. J., Stark, B. C. 1989. Expression of β-Lactamase by Recombinant *Escherichia coli* Strains Containing Plasmids of Different Sizes-Effects of pH, Phosphate, and Dissolved Oxygen. Biotechnol. Bioeng. **34**: 309.
25. Stark, M. J. R. 1987. Multicopy Expression Vectors Carrying the *lacI* Repressor Gene for Regulated High-Level Expression of Genes in *Escherichia coli.* Gene. **51**: 255.
26. Wood, T. K., Kuhn, R. H., Peretti, S.W. 1990. Enhanced Plasmid Stability Through Post-Segregational Killing of Plasmid-Free Cells, Biotechnol. Technol. **4**: 36.
27. Wood, T. K., Peretti, S.W. 1990. Depression of Protein Synthetic Capacity Due to Cloned-Gene Expression in *E. coli.* Biotechnol. Bioeng. **36**: 865.
28. Wood, T. K., Peretti, S.W. Effect of Specialized Ribosomes on Recombinant Protein Synthesis in *E. coli.* Biotechnol. Bioeng., in press.