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Feedback regulation of rRNA and tRNA synthesis and accumulation of free ribosomes after conditional expression of rRNA genes

(gene fusions/λ P_L promoter/guanosine tetraphosphate)

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ABSTRACT **We have constructed a conditional rRNA gene expression system by fusing a plasmid-encoded *rrnB* operon to the λ P_L promoter/operator. It was thereby possible to study the events that lead to the regulation of chromosomal rRNA and tRNA synthesis after overproduction of rRNA. rRNA induction resulted in a 2-fold increase in 30S and 50S free ribosomal subunits, whereas the polysome fraction was unaffected. Overproduction of rRNA and "free" ribosomes produced a large repression of rRNA and tRNA synthesis from chromosomal genes and a smaller increase in the concentration of guanosine tetraphosphate. These results lend support to the ribosome feedback regulation model: rRNA and tRNA operons are negatively regulated, either directly or through some intermediate, by free, nontranslating ribosomes.**

We have recently proposed a model to explain the growth-rate-dependent control of rRNA and tRNA synthesis in *Escherichia coli* (ref. 1; for detailed discussion and earlier related studies, see also ref. 2). Utilizing plasmids containing intact rRNA genes, we showed that total rRNA synthesis is not significantly affected by an increase in gene dose, because individual rRNA operons are proportionally repressed. On the other hand, the presence of extra *defective* rRNA genes causes a gene-dose-dependent increase in the total transcription rate of rRNA (defective plus intact) (1, 3). We suggested that surplus "free" ribosomes, assembled but not engaged in protein synthesis, act as feedback repressors to limit rRNA and tRNA synthesis, either directly or through some intermediary effector.

To study further the mechanism of the negative feedback regulation of rRNA and tRNA synthesis, we have now constructed a conditional expression system for the production of rRNA by substituting the λ P_L promoter/operator for the normal promoters of a plasmid-encoded rRNA operon. Such a system makes possible the analysis of the events taking place between the overproduction of rRNA and the ultimate result, the inhibition of transcription from individual chromosomal rRNA and tRNA operons. The results presented here, obtained with this new system, further support the proposed ribosome feedback regulation model.

MATERIALS AND METHODS

Plasmids were constructed as described in Fig. 1. C600 (λ*c*I857, *cro*27, *S7*), provided by W. Szybalski, was used as a host strain for constructions involving λ P_L plasmids. All physiological experiments were carried out in the strain NO3203, a *recA* derivative of M5219. M5219 is *lacZ*(am), *trp*(am), *strA*, *uvrB*, *bio* and carries a defective prophage λ*bio*252*c*I857ΔHI, which provides a temperature-sensitive repressor (4). Other methods are described in the figure and table legends.

RESULTS

Plasmid Constructions. We have constructed a conditional expression system for overproduction of rRNA *in vivo*. A DNA fragment containing the λ P_L operator/promoter was fused to a DNA fragment containing the entire *rrnB* operon but lacking both rRNA promoters *P1* and *P2*. The fused operon (called "P_L-*rrnB*" in this paper) is located between the *Hin*dIII and *Bam*HI sites of pBR322 (Fig. 1; for details, see legend). P_L-*rrnB* contains (*i*) λ DNA including the intact P_L operator/promoter up to but *not* including the base corresponding to the transcription start site, (*ii*) a *Kpn* I oligonucleotide linker, and (*iii*) the complete *rrnB* DNA region starting 3 base pairs (bp) downstream of the transcription start site for *P2*. Therefore, the DNA regions coding for virtually the entire rRNA precursor (including the RNA processing sites and the presumed antitermination elements; refs. 8 and 9) are present in the fusion operon. A derivative of the fusion operon was then constructed to serve as a control plasmid by deleting a large *Sst* II fragment containing the distal 2/3 of 16S rRNA, the spacer tRNA, and the proximal 2/3 of 23S rRNA genes. This deletion derivative (called "P_L-Δ*Sst*" in this paper), therefore, cannot make functional rRNA products.

Plasmids were maintained in a strain carrying a defective λ prophage that contains the temperature-sensitive *c*I857 allele of the λ repressor gene. The λ *c*I857 repressor is inactivated at 42°C, but at 30°C it represses transcription from P_L completely. Cells carrying the control plasmid with P_L-Δ*Sst* show no significant differences from those carrying pBR322 in the synthesis of rRNA and tRNA at least for several hours after "induction" (data not shown).

Growth Characteristics. The strains containing the plasmids with P_L-*rrnB* or P_L-Δ*Sst* grow identically at 30°C in minimal glucose medium. However, after about 2 hr at 42°C, growth rate differences become noticeable between the two strains (Fig. 2*A*), with the strain carrying the P_L-*rrnB* plasmid growing significantly more slowly. All of the experiments to be described below were performed during the time period after the temperature shift when the two strains were growing at the same rate.

RNA Accumulation. Fig. 2*B* shows that the strain with the P_L-*rrnB* fusion has the same RNA-to-cell-mass ratio as the control strain with P_L-Δ*Sst* at 30°C, but this ratio increases by about 15% within 30 min after the shift to 42°C and remains at this value during the period measured. Although extra RNA accumulation in the strain with P_L-*rrnB* is modest relative to the increase in the rRNA synthesis rate (see below), it is sufficient to account for the 2-fold increase in

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Abbreviations: bp, base pair(s); ppGpp, guanosine tetraphosphate.

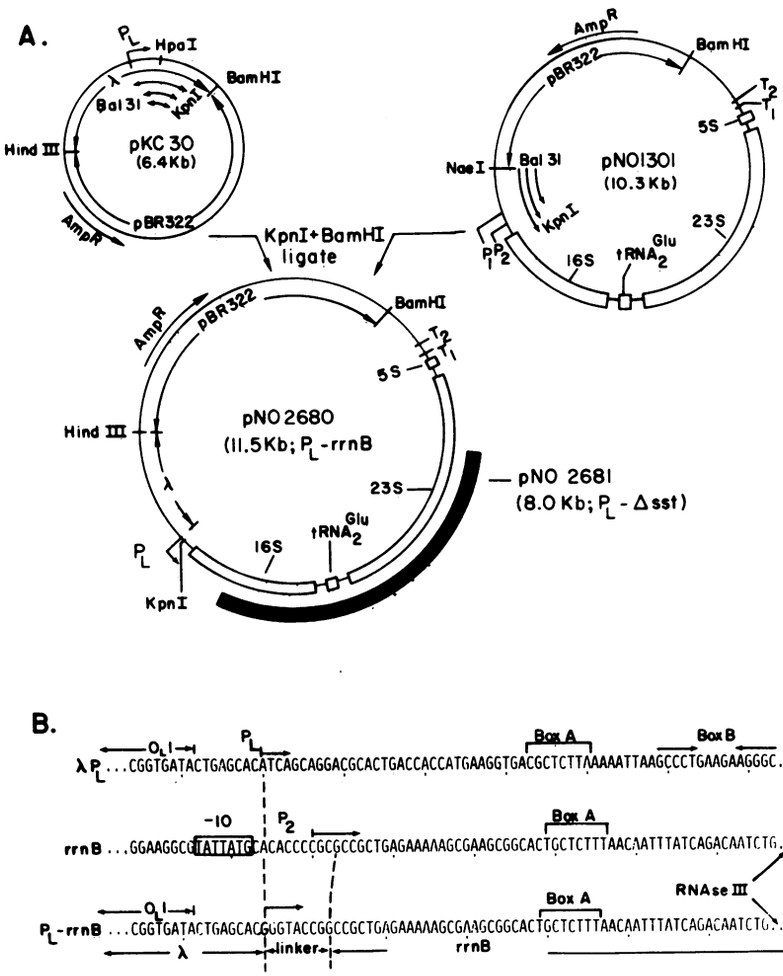

FIG. 1. (*A*) Construction of P_L-*rrnB* and P_L-Δ*Sst*. It was necessary to delete λ *nutL* from pKC30 (5) because the presence of this sequence was found to have inhibitory effects on host rRNA synthesis (unpublished experiments). Plasmid pKC30 (provided by M. Gribskov) was digested with *Hpa* I, treated with exonuclease BAL-31, and then ligated in the presence of a *Kpn* I linker (G-G-G-T-A-C-C-C; New England Biolabs no. 1016). After transformation of C600 (λ) with this DNA preparation, a plasmid (pNO2678) with an appropriate-sized deletion was selected. The deletion in pNO2678 begins at the first base of the P_L transcript and extends downstream 631 bp as determined by DNA sequencing (6). A series of *rrnB* plasmids deleting various amounts of DNA from the promoter region was constructed by exonuclease BAL-31 digestion of pNO1301 (1, 7) starting at the unique *Nae* I site, 714 bp upstream of the *P2* start site, followed by ligation in the presence of *Kpn* I linkers (C-G-G-T-A-C-C-G; New England Biolabs no. 1023). Before transformation, *Kpn* I–*Bam*HI fragments from the exonuclease-treated plasmids were ligated into *Kpn* I plus *Bam*HI-digested pNO1301 in which a *Kpn* I linker had been inserted at the *Nae* I site. Consequently, the resulting transformants contained deletions that extended in one direction only from the original *Nae* I site. One of the transformants (pNO1342) had a deletion covering both the *P1* and *P2* promoters, extending to the third base downstream of the *P2* transcription start site. The plasmid pNO2680 carrying the fusion operon, P_L-*rrnB*, was constructed by ligation of *Kpn* I plus *Bam*HI digests of pNO2678 and pNO1342. The plasmid pNO2681 carrying a defective fusion operon (P_L-Δ*Sst*) was constructed by *Sst* II digestion of pNO2680 followed by ligation and transformation. Solid bar = *Sst* II deletion. Open bars = rRNA coding regions. kb, Kilobases. (*B*) DNA sequences of the P_L region of pKC30, the *rrnB P2* promoter region of pNO1301, and of the fusion operon P_L-*rrnB*. The operator/promoter region from λ P_L, the −10 region from *rrnB P2*, the RNase III cleavage site in *rrnB*, the "box A" and "box B" sequences required for transcription antitermination in the λ *nutL* region (8), and inferred RNA transcription start sites are indicated on the sequences.

the accumulation of free nontranslating ribosomes relative to the control strain observed at 42°C (see below).

Free Ribosome Accumulation. We measured amounts of polysomes, monosomes, and 50S and 30S subunits before and after induction of the transcription of P_L-*rrnB*. The strains with P_L-*rrnB* or P_L-Δ*Sst* were grown in a medium containing [^{32}P]orthophosphate for several generations at 30°C, and the cultures were then shifted to 42°C. Cells were harvested before and 30 min after the shift, mixed with a constant amount of [^{3}H]uridine-labeled reference cells, and then gently broken by using a lysozyme-freeze-thaw method. The polysome/ribosome profiles from the two strains were examined by sucrose gradient centrifugation.

Fig. 3 *A* and *B* shows the polysome/ribosome profiles in the two strains 10 min before the shift to 42°C. Relative to the profile of the ^{3}H-labeled reference polysome/ribosomes, there is no obvious difference between the two strains. Quantitation revealed no more than a 5% difference in the amounts of polysomes, monosomes, or free ribosomal subunits between the two strains.

Fig. 3 *C* and *D* shows polysome/ribosome profiles 30 min after the temperature shift. It is clear that there is an increase in the amounts of both free 50S and 30S ribosomal subunits from the strain with P_L-*rrnB*. Quantitation revealed that the ratio of the amounts of free 50S and 30S subunits from the P_L-*rrnB* strain relative to the control strain is about 2.0, whereas the ratios of the amounts of polysomes and monosomes are both about 1.0. The same results were also obtained with samples taken at 90 min after the temperature shift (data not shown). Thus, induction of rRNA transcription from the P_L promoter results in a 2-fold increase in the amounts of free 50S and 30S subunits (but not in the amount

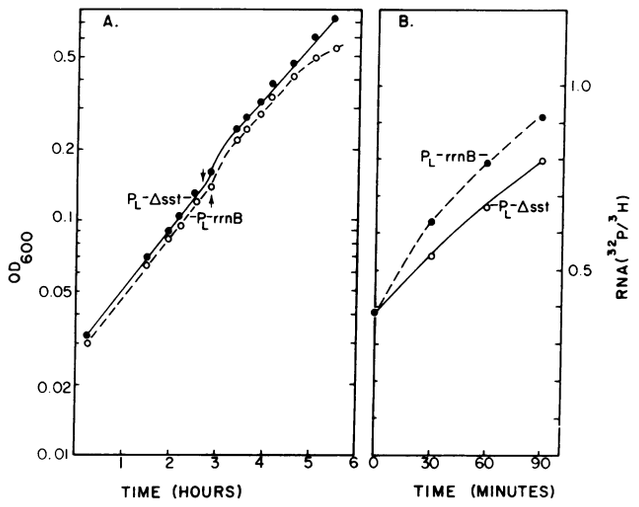

FIG. 2. Growth and RNA accumulation in strains containing P_L-*rrnB* or P_L-Δ*Sst*. (*A*) Cells carrying either the P_L-*rrnB* plasmid or the P_L-Δ*Sst* plasmid were grown for several generations at 30°C in MOS medium (10) containing 0.3 mM K_2HPO_4, 50 μg of tryptophan per ml, 1 μg of thiamine per ml, 1 μg of biotin per ml, 0.4% glucose, and 100 μg of ampicillin per ml. Cells were then shifted to 42°C (arrows). Cell density was followed by measuring OD at 600 nm (OD_{600}). (*B*) Cells were grown in the above medium containing 10 μCi of [^{32}P]orthophosphate per ml (1 Ci = 37 GBq) for several generations. At an OD_{600} of 0.14, the cells were shifted to 42°C. At indicated times after the shift, samples were taken and mixed with a constant amount of reference cells that had been labeled with [^{3}H]uridine, and RNA was prepared by phenol extraction followed by ethanol precipitation. The amounts of [^{32}P]RNA and [^{3}H]RNA in this RNA preparation were measured. The ratios of ^{32}P to ^{3}H shown represent the relative amounts of cellular RNA per unit volume of culture.

of polysomes) by 30 min, and this level of overproduction is maintained for at least another 60 min. It should be noted that the two strains grow at the same rate for about 2 hr after the temperature shift (see above and Fig. 2*A*), and direct measurements of total protein synthesis rates showed only slight differences between the two strains during this time period. The fact that the rate of protein synthesis and the levels of polysomes are nearly identical in the two strains after induction suggests that peptide chain elongation rates are identical and that total protein synthesis rates are a direct function of polysome levels. We also note that the 70S monosome fractions analyzed very likely contain large amounts of "monosomes" produced by polysome breakdown during and/or after cell breakage. We feel that it may be technically difficult to determine the amounts of free 70S ribosomes that are not engaged in protein synthesis.

Inhibition of rRNA and tRNA Synthesis. The accumulations of the tRNAs encoded by chromosomal rRNA operons and tRNA operons were measured after induction of transcription from P_L-*rrnB* in order to quantitate the inhibition of rRNA and tRNA synthesis from individual chromosomal operons.

Cells containing the P_L-Δ*Sst* plasmid or the P_L-*rrnB* plasmid were grown to the same cell density, shifted to 42°C, and then labeled with [^{32}P]orthophosphate for the time interval 30–90 min after the shift. The amounts of various tRNAs synthesized during this time interval were determined for the P_L-*rrnB* strain relative to the control strain, and the results are shown in Table 1. First, $tRNA_2^{Glu}$ was highly overproduced. This was expected since the gene for this spacer tRNA is carried by P_L-*rrnB*. Second, tRNAs encoded within chromosomal rRNA operons and tRNA operons are repressed by an average of almost 60% in the strain with P_L-*rrnB* as compared to the control strain.

rRNA Synthesis Rates. Using the data from Table 1 and assuming that accumulations of the spacer tRNAs reflect rRNA synthesis rates (because tRNAs are stable), we can calculate indirectly the relative synthesis rates of rRNA in the two strains after induction. If the control strain has an rRNA synthesis rate of 7 arbitrary units (from four operons containing $tRNA_2^{Glu}$ genes and three containing $tRNA_1^{Ile}$ and $tRNA_{1B}^{Ala}$ genes; see ref. 13), then the strain with P_L-*rrnB* has a synthesis rate of 15.2 (i.e., 4 × 3.5 + 3 × 0.4). Therefore, the strain with P_L-*rrnB* must have synthesized rRNA at a rate ≈2-fold that of the control strain (15.2/7 = 2.2).

This increase in synthesis rate has been confirmed (data not shown) by measuring the instantaneous rRNA synthesis rates of the two strains at 30 min after induction, using a method described previously (1). Since, as shown in Fig. 2*B*, total rRNA *accumulation* increased only slightly (about 15% during the 30- to 90-min time interval after induction), it is clear that most of the newly synthesized rRNA must not be stable. Presumably, the synthesis rates of at least some ribosomal proteins are not sufficient to keep up with the increase in rRNA synthesis and, therefore, the extra rRNA is not protected from degradation by assembly into ribosomes. [We have found that the synthesis of ribosomal protein *mRNA* is inhibited after induction of P_L-*rrnB* (unpublished experiments; see also ref. 2). This might explain why ribosomal protein synthesis rates are not sufficient despite the fact that translational control mechanisms increase ribosomal protein synthesis when rRNA synthesis increases.]

Table 1. Accumulations of tRNAs and 5S RNAs from strains containing the P_L-*rrnB* or P_L-Δ*Sst* plasmid

Group	RNA	P_L-*rrnB*/P_L-Δ*Sst*
I	5S	0.68
	5SIII	0.64
II	Glu 2	3.50
	Trp	0.42
	Ala 1B	0.40
	Ile 1	0.41
	Asp 1	0.37
III	Leu 1	0.45
	Ser 3	0.41
	Ser 1	0.51
	Tyr 1 + 2	0.47
	Asn/Met	0.50
	Phe	0.47
	Gly 3	0.43
	Val 1	0.43
	His	0.41
IV	1 (λ transcript)	0.98
	2	1.01
	3	1.02
	4	0.94

Cells were grown at 30°C as in Fig. 2 and shifted to 42°C at an OD_{600} of 0.14. [^{32}P]Orthophosphate (100 μCi/ml) was added at 30 min after the shift and incubation was continued for an additional 1 hr. One-half milliliter of cells was then mixed with an equal amount of reference cells (labeled with [^{3}H]uridine). RNA extraction, separation of tRNAs by two-dimensional gel electrophoresis, and quantitation of the [^{32}P]RNA and [^{3}H]RNA have been described (1). The values ($^{32}P/^3H$) for the individual tRNA and non-tRNA spots represent their relative amounts. The ratios of these values obtained for the strain with P_L-*rrnB* to the corresponding ones for the control P_L-Δ*Sst* strain are shown in the table. "5SIII" is a minor form of 5S RNA encoded by *rrnA* (12). Group II comprises tRNAs encoded in rRNA operons. Other tRNAs analyzed that are not encoded by rRNA operons are shown in group III. RNA spots that are neither tRNA nor 5S RNA were also analyzed and the values are given in group IV. The "λ transcript" refers to a transcript seen in strains harboring λ prophages and plasmids carrying the P_L *nutL* region (data not shown).

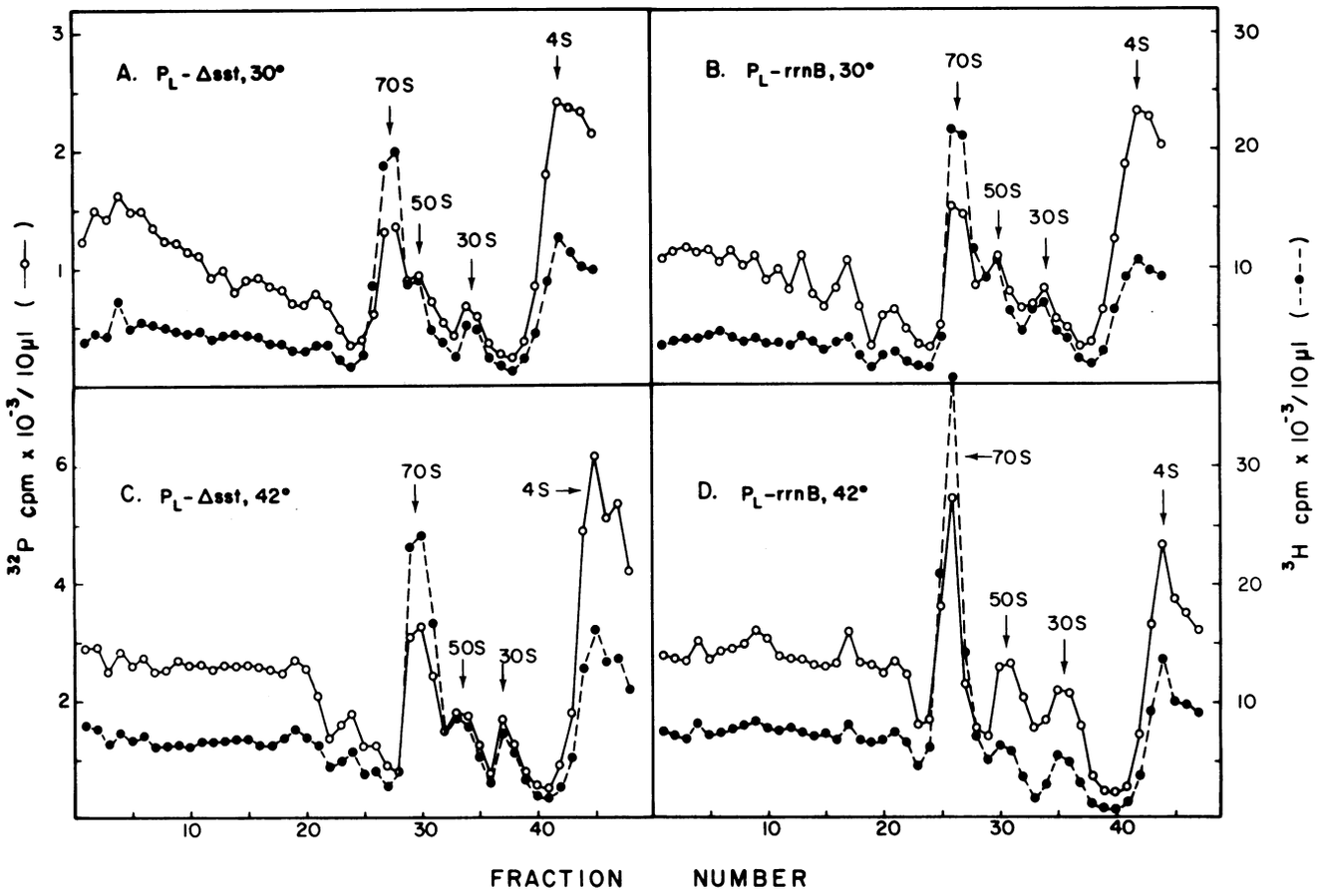

FIG. 3. Polysome/ribosome analysis of cells containing the P_L-*rrnB* or P_L-Δ*Sst* plasmid. Cells containing P_L-Δ*Sst* (*A* and *C*) or P_L-*rrnB* (*B* and *D*) were grown at 30°C in the medium described in the legend to Fig. 2 supplemented with [^{32}P]orthophosphate (20 μCi/ml). At an OD_{600} of 0.14, the cultures were transferred to 42°C. Samples (5 ml) were taken at 10 min before (*A* and *B*) and at 30 min after (*C* and *D*) the temperature shift, mixed with 5 g of ice and 100 μl of 1 M sodium azide, and cooled quickly to 0°C by vigorous shaking in a dry ice/acetone bath. The samples were then mixed with ^{3}H-labeled reference cells (see below) at 4°C and centrifuged at 4°C, and the pellet was suspended in 1 ml of lysis buffer [5 mM Tris·HCl, pH 7.5/5 mM $MgSO_4$/60 mM KCl/18% (wt/vol) sucrose/10 mM NaN_3/300 μg of lysozyme per ml; ref. 11]. The cell suspensions were centrifuged at 4°C, and the pellets were frozen in liquid nitrogen. The frozen pellets were resuspended in 25 μl of lysis buffer on ice, and cells were lysed by repeated freezing and thawing, followed by addition of 75 μl of buffer (5 mM Tris·HCl, pH 7.5/5 mM $MgSO_4$/60 mM KCl), 10 μl of 5% Brij 58 (wt/vol), and 2 μl of DNase I (DPFF, Worthington) at 1 mg/ml at 0°C. After 10 min, samples were spun for 15 sec at 4°C in a Microfuge, and supernatants were placed on 5-ml, 14–40% (wt/vol) sucrose gradients (in 25 mM Tris·HCl, pH 7.5/60 mM KCl/10 mM $MgCl_2$) and centrifuged in an SW 50.1 rotor at 45,000 rpm for 80 min at 4°C. [^{32}P]RNA and [^{3}H]RNA in each fraction were precipitated with 5% trichloroacetic acid, filtered, and assayed for radioactivity. ^{3}H-labeled reference cells were prepared by growing cells for four generations in the presence of [^{3}H]uridine (20 μCi/ml). Fifty milliliters of culture was quickly cooled as described above and centrifuged at 4°C. Pellets were suspended in 5 ml of lysis buffer without lysozyme, distributed in 0.5-ml aliquots, quickly frozen in liquid nitrogen, and stored at −70°C.

Accumulation of Guanosine Tetraphosphate (ppGpp). It is possible that the regulation of rRNA and tRNA operons by free ribosomes does not involve a direct physical interaction between ribosomes and DNA and/or RNA polymerase but takes place via some intermediary effector(s). Since there is evidence that ppGpp levels correlate inversely with rRNA and tRNA synthesis rates (e.g., refs. 14 and 15), ppGpp might be a regulatory intermediate. We have therefore measured ppGpp concentrations before and after the induction of the transcription of P_L-*rrnB* and compared them with those in the control strain with P_L-Δ*Sst*. The results are shown in Fig. 4.

As expected, ppGpp levels rose in both strains after the temperature increase (e.g., ref. 17). However, the level of ppGpp after induction was significantly (30–50%) higher in the strain with P_L-*rrnB* than in the control strain, and this difference was noticeable at 10 min as well as at later times (30 and 60 min) after induction. In contrast, ATP and GTP levels were virtually identical in both strains before and after the induction.

DISCUSSION

The experimental system for the conditional expression of rRNA genes described in this paper has advantages over the system used in the previous studies (1, 3). The new system makes it possible to overproduce the rRNA from a specific rRNA operon, measure the entry of rRNA into free ribosomes, and separately monitor the effects on the repression of chromosomal rRNA and tRNA synthesis. The experimental results obtained with the present system, together with the previous results (1, 3), clearly establish a causal relationship between the excess synthesis of rRNA and ribosomes and the inhibition of rRNA and tRNA synthesis and strongly support the ribosome feedback regulation model. In particular, the previously postulated increase in the amounts of free nontranslating ribosomes has now been demonstrated directly as a result of the induction of the synthesis of excess rRNA.

Induction of excess rRNA synthesis from the P_L promoter leads to the inhibition of transcription of rRNA genes on the

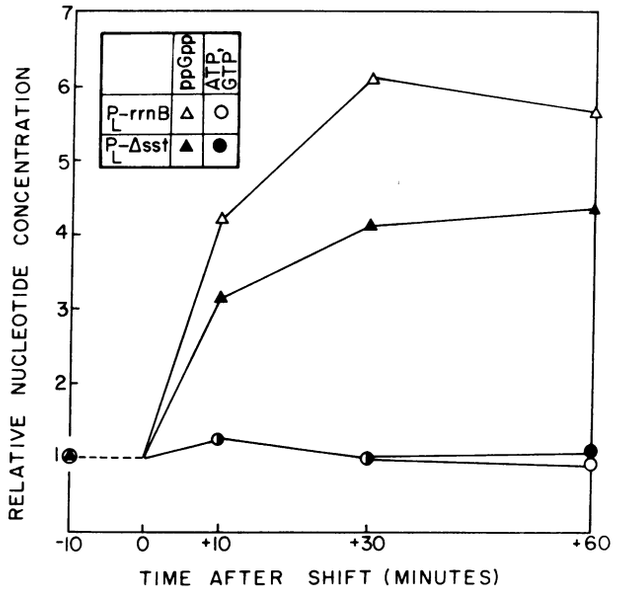

FIG. 4. Nucleotide levels in strains containing the P_L-*rrnB* or P_L-Δ*Sst* plasmid before and after a shift from 30°C to 42°C. The amounts of nucleotides per unit amount of cells are expressed as ^{32}P:^{3}H ratios divided by cell density (OD_{600}) and then normalized to the values of the strain containing the P_L-Δ*Sst* plasmid at 30°C (= 1.0). Symbols are indicated in the *Inset*. Cells were grown as described in the legend to Fig. 3 in the presence of [^{32}P]orthophosphate (80 μCi/ml) at 30°C and shifted to 42°C at an OD_{600} of 0.2 (time 0). At the times indicated, 100-μl samples were placed into 10 μl of 11 M formic acid and kept on ice for 30 min. An aliquot of [^{3}H]guanosine-labeled control formic acid extract (see below) was added to each sample, orthophosphate was removed by precipitation (16), and nucleotides were separated by two-dimensional thin-layer chromatography (16). The ATP, GTP, and ppGpp spots were localized by autoradiography, cut out, and placed in 1 ml of 1.5 M NH_4OH. After 30 min at room temperature, 0.8 ml was removed and mixed with 0.7 ml of H_2O, and ^{32}P and ^{3}H were counted in 10 ml of Aquasol. The ^{3}H-labeled reference formic acid extract was prepared by growing strain LL309 (Rel^+) at 37°C in the same medium to an OD_{600} of 0.3. Carrier-free [^{3}H]guanosine (100 μCi) was added to 1 ml of cells and 5 min thereafter 1.5 mg of serine hydroxamate per ml was added, and incubation was continued for 4 min. Cells were centrifuged and resuspended in 200 μl of medium containing serine hydroxamate, and 20 μl of 11 M formic acid was added. After 30 min on ice, the extract was frozen at −70°C.

chromosome. By using the data shown in Table 1, one can calculate that about 80% of the total rRNA synthesis is from P_L-*rrnB* on the plasmid and the rest is from the native promoters on the chromosome. Other unpublished experiments suggest that the *rrnB* region contained on the fusion operon, P_L-*rrnB*, does not contain the target site for the feedback regulation by free ribosomes. This, combined with the inherent strength of the P_L promoter, most likely explains the high levels of rRNA gene transcription from the fusion operon observed under these conditions.

We have observed that the cellular concentration of ppGpp is elevated after the induction of excess rRNA synthesis from P_L-*rrnB* compared to the control strain carrying P_L-Δ*Sst*. Although the difference is not particularly large (30–50% over the control strain) compared to the degree of inhibition of chromosomal rRNA and tRNA synthesis (2-fold), it is possible that ppGpp is directly involved in the inhibition of the transcription of the chromosomal rRNA and tRNA operons as suggested in earlier studies (for reviews, see refs. 2, 18, and 19). However, it is also possible that the increase in ppGpp is the result rather than the cause of inhibition of chromosomal rRNA and tRNA transcription. The third possibility is that both the increase in ppGpp and the inhibition of rRNA and tRNA transcription are the results of induction of excess synthesis of rRNA (and ribosomes) but are independent events without any causal relationship. At the moment, we cannot distinguish among these three possibilities.

The induction of excess rRNA synthesis in the present system led to a 2-fold increase in the amount of free ribosomes (detected as free 30S and 50S ribosomal subunits) without a detectable increase in the amount of polysomes. This specific increase was caused without changing nutritional conditions, growth rate, or protein synthesis rate (relative to the control strain). Therefore, the entry of free (nonfunctioning) ribosomes into functioning polysomes under these conditions must be determined *not* by the concentration of free ribosomes but by the rate of mRNA synthesis (and degradation) and/or other factors such as those regulating the rate of initiation of translation directly. It is expected that total protein synthesis rates would be determined in some complex way by nutritional conditions.

Finally, we note that the present system makes it possible to study the effects of increased free ribosome concentrations on transcription from other promoters, such as ribosomal protein promoters. The repressible nature of the system also allows the isolation of mutations in rRNA and possibly ribosomes that would otherwise be lethal (e.g., see ref. 20). These analyses, as well as more detailed analyses of the biochemical mechanisms involved in the regulation of rRNA and tRNA synthesis, await further investigation.

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