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SYNTHESIS OF NON-TRANSLATING OR TRANSLATING SPECIALIZED RIBOSOMES CAUSES FEEDBACK REGULATION OF RIBOSOMAL RNA SYNTHESIS IN *Escherichia coli*

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Specialized ribosomes carry a mutant anti-Shine-Dalgarno region that disrupts the complementary base pairing that stabilizes the translation initiation complex with *E. coli* mRNAs. It has been reported that production of specialized ribosomes does not cause the inhibition of chromosomal rRNA synthesis that follows production of wild-type ribosomes. We proposed that enabling translation on specialized ribosomes by providing mRNA with a complementary mutation in the Shine-Dalgarno region would restore feedback regulation and inhibit chromosomal rRNA synthesis. With both our system and the system studied previously, we saw feedback regulation regardless of whether the specialized ribosomes were translating. As reported previously, transcription from plasmid-borne promoters decreased as chromosomal rRNA synthesis was repressed, suggesting that the λP_L and *tac* promoters may be sensitive to the effector(s) of feedback regulation.

Evidence that synthesis of rRNA in *E. coli* is regulated by a feedback mechanism initially pointed to free, non-translating ribosomes as a likely effector. The total rRNA synthesis rate is nearly independent of gene dosage; transcription from a plasmid-borne rRNA operon represses transcription from chromosomal rRNA operons (1). When the plasmid-borne operon encodes rRNA that cannot be incorporated into functional ribosomes, the total rRNA transcription rate increases in proportion to gene dosage (1). These results suggest that regulation of rRNA transcription is a feedback response that depends on the assembly of functional ribosomes.

More direct evidence for this hypothesis was obtained with a conditional rRNA expression system (2). Induction of rRNA synthesis from the plasmid-borne operon resulted in a substantial increase in the number of 30 S and 50 S subunits, but no increase in polysomes. At the same time, induction repressed chromosomal synthesis of rRNA. These observations are consistent with the notion of free, non-translating ribosomes (or subunits) as feedback regulation effectors.

Specialized ribosomes (3) allowed a definitive test of this hypothesis. These ribosomes carry a mutation in the ASD region of 16 S rRNA that abolishes the complementary base pairing that

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Abbreviations: SD, Shine-Dalgarno; ASD, anti-Shine-Dalgarno; OD_{600}, optical density at 600 nm.

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stabilizes the translation initiation complex with *E. coli* mRNAs. Synthesis of specialized ribosomes creates a pool of free, non-translating ribosomes that would, it was assumed, strongly repress rRNA synthesis from the chromosome. However, this experiment showed that there was virtually no inhibition of chromosomal rRNA synthesis (4); non-translating specialized ribosomes did not participate in feedback regulation. It appeared that the feedback effector was related not to free, non-translating ribosomes but instead to ribosomes capable of initiating translation.

We proposed that enabling translation on specialized ribosomes would restore the feedback regulation observed upon induction of the synthesis of wild-type ribosomes, and we tested this hypothesis by providing specialized mRNA to the specialized ribosome system.

Materials and Methods

Plasmids and bacterial strains

The plasmids used in this work are shown in Figure 1. On the plasmids used by Yamagishi *et al.* (4) (Fig. 1A, 1B), transcription of the rRNA operon (*rrnB*) is regulated by the λP_L promoter and the temperature-sensitive *cI857* repressor (supplied by the host cell). The control plasmid carries a deletion comprising the distal end of the 16 S rRNA gene, the spacer $tRNA_2^{Glu}$ gene, and the proximal end of the 23 S rRNA gene. These plasmids, in the host strain NO3203 [*lacZ*(am), *trp*(am), *strA*, *uvrB*, *bio*, λ*bio252cI857ΔHI*] (4), were kindly provided by Masayasu Nomura. On our rRNA plasmids (Fig. 1C), transcription of *rrnB* is regulated by the *tac* promoter. Our control plasmid carries a deletion identical to that of the control used by Yamagishi *et al.* (4).

The plasmid providing specialized mRNA (Fig. 1D) is a derivative of pOU79 (5). The copy number is regulated by the λP_R promoter and the *cI857* repressor; the gene for the repressor is carried on the plasmid. The SD region of the gene for a ß-galactosidase fusion protein, transcribed from a constitutive promoter, was modified to be complementary to the ASD mutation in the specialized rRNA. For the control plasmid, the promoter and 5' end of the fusion gene were deleted.

Our plasmids were maintained in the host strain JM109 [*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), {F' *traD36*, *proAB*, $lacI^qZ\Delta M15$}] (6), obtained from Stratagene. Construction of these plasmids has been described elsewhere (7).

The basal growth medium was MOPS medium (8) supplemented with 4 g/l glucose, 1 μg/ml thiamine, and 100 μg/ml ampicillin; the phosphate concentration was reduced to 0.33 mM. For the first experiment with the NO3203 strains, this basal medium was supplemented with 40 μg/ml each L-amino acid except proline and 1 μg/ml biotin. For the second experiment with the NO3203 strains, the basal medium was supplemented with 50 μg/ml tryptophan and 1 μg/ml biotin, as described by Yamagishi *et al.* (4). For the JM109 strains, the basal medium was supplemented with 40 μg/ml each L-amino acid except proline and 12.5 μg/ml tetracycline.

Radioactive labeling of cells

^{3}H-labeled reference cells (2) were prepared by growing JM109/pRJL1-Δ*Sac*II/pLOU1-Δ*copB*p for 3-5 h in medium containing 20 μCi/ml [5,6-^{3}H]uridine (New England Nuclear). Labeled cells were recovered by centrifugation, resuspended in 1-2 ml medium containing 10% glycerol, and stored in 100 μl aliquots at -70°C.

^{32}P-labeled cells were prepared by inoculating fresh medium from overnight cultures to an OD_{600} of 0.05-0.15 and incubating at 30°C (NO3203) or 37°C (JM109) and 300 rpm for 2-3 h. Transcription from the plasmid-borne rRNA operon was induced by transferring the tubes to a shaking water bath equilibrated at 42°C (NO3203) or by adding IPTG to 1 mM (JM109). At 15-20 min (NO3203) or 15-40 min (JM109) after induction, 50 μCi [^{32}P]orthophosphoric acid (New England Nuclear) was added to 0.5 ml culture and incubation was continued for 40 min (NO3203) or 30-85 min (JM109). The ^{32}P-labeled cells were added to an aliquot of the ^{3}H-labeled reference cells and RNA was recovered essentially as described (9).

The JM109 strains making specialized ribosomes generally grew more slowly than did the strains making only wild-type ribosomes. The *tac* promoter in these strains is not completely repressed, even in the absence of induction, and the significant fraction of specialized ribosomes in the uninduced strains (15%) is sufficient to reduce the growth rate (7). Therefore, we typically labeled the specialized ribosome strains for a longer time than we labeled the wild-type ribosome strains to get approximately the same cell density change during labeling for each strain.

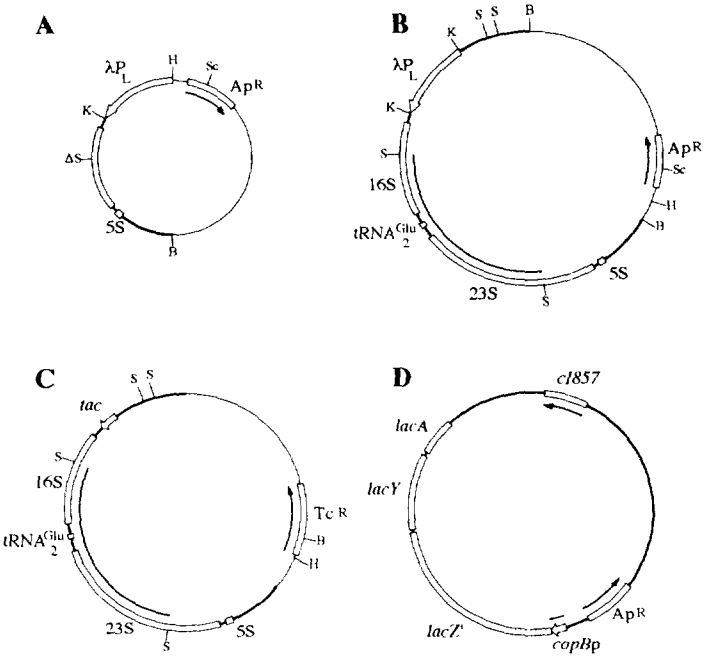

Figure 1. Plasmids used in this work. (A) P_L-Δ*Sst* (pNO2681) is the control plasmid used in previous regulation studies (2, 4). Induction by temperature shift-up does not lead to feedback regulation of rRNA synthesis (2). (B) pASD(VIII,X)-P_L carry intact *rrnB* operons with either wild-type (ASDVIII = CCTCC) or mutant (ASDX = CTCTC) ASD sequences. Although P_L-Δ*Sst* and pASD(VIII,X)-P_L carry the same P_L-*rrnB* fusions, these fusions have opposite orientations with respect to the pBR322 vector sequences. The *Sst*II deletion in P_L-Δ*Sst* is indicated by the inside heavy line. (C) pRJL1-ASD(VIII,X) carry ASD regions identical to those of pASDVIII-P_L and pASDX-P_L, respectively. The control plasmid pRJL1-Δ*Sac*II carries the *Sac*II deletion shown by the inside heavy line. This deletion is identical to the *Sst*II deletion of P_L-Δ*Sst* (*Sac*II and *Sst*II are isoschizomers). (D) pLOU1-SDX carries a *lacZ* gene truncated at the 5' end and fused in frame to the 5' end of the *copB* gene, which is transcribed from its constitutive promoter. The SD region (SDX = GAGAG) of this fusion gene has been modified to be complementary to ASDX, providing specialized mRNA for translation on the specialized ribosomes. The control plasmid pLOU1-Δ*copB*p is deleted of the *copB* promoter and the 5' end of the *copB* gene as shown by the inside heavy line. This deletion also introduced a stop codon immediately preceding and in-frame with the remainder of the *lacZ* gene. In (A)-(C), thin lines are pBR322 sequences; several restriction sites are shown to indicate structural relationships between the plasmids. B=*Bam*HI, H=*Hin*dIII, K=*Kpn*I, S=*Sst*II (or *Sac*II), Sc=*Sca*I.

Two-dimensional gel electrophoresis of tRNA

Two-dimensional gel electrophoresis was carried out essentially as described (1), except that all electrophoresis was done at room temperature instead of at 15°C. Individual tRNAs were excised from the gel, transferred to scintillation vials, and minced. After adding 0.5 ml RNase A (50 μg/ml) to each vial, the samples were vortexed and incubated for at least 6 h at 37-42°C. After the RNase digestion, 5 ml ScintiVerse II (Fisher) was added and the samples were shaken vigorously. Radioactivity in the samples was measured on a Beckman LS-5000TA scintillation counter.

Individual tRNAs were identified by resolving unlabeled tRNAs by the same electrophoretic procedure, staining the second-dimension gel with ethidium bromide, and excising the individual spots. The 5' phosphates were removed with bacterial alkaline phosphatase and replaced with ^{32}P phosphates; these end-labeled tRNAs were sequenced by partial digestion with sequence-specific RNases (RNA Sequencing System, BRL).

Results

Identification of individual tRNAs

As in previous studies (1, 2, 4, 10), we monitored the synthesis of tRNA as a marker for rRNA synthesis. tRNA and rRNA synthesis is co-regulated (1), but excess rRNA that is not incorporated into ribosomes can be degraded (2), whereas newly synthesized tRNA is stable (11). Although there is considerable evidence that all tRNA synthesis is coordinately regulated (1, 2, 4, 10), we decided to identify some of the tRNAs for two reasons: to ensure that the spots we saw on our second-dimension gel were actually tRNAs and to confirm the identity of the Glu2 spot (we had tentatively identified Glu2 from its position on the gel and its response to induction). Sequence data from individual spots were compared to sequences of *E. coli* tRNA genes (12) as shown in Table 1. Five spots were identified as specific tRNAs (including Glu2); seven others that were not unequivocally identified were also included in our analysis.

tRNA abundances

The average values for tRNA abundances are summarized in Table 2 and compared to the results of Yamagishi *et al.* (4). Corrections are incorporated for background subtraction, spillover of ^{32}P counts to the ^{3}H channels, ^{32}P decay, and cell density. The net effect of these corrections is generally quite small since the corrections are applied to both the numerator (experimental strain) and denominator (control strain) of the final ratio. The cell density correction is significant in accounting for the different growth rates of the JM109 strains (see **Materials and Methods**).

Table 1. Identification of individual tRNAs by sequencing

spot #4	sequence data [a] gene sequence [b] mismatch? [c]	GCGCUACA?GCU-AGCCCGGGAGAGUG?UUG GGGCTATA-GCTCAGCt-GGGAGAGCGCCTG * *(*) (*) (**) * *	Ala2
spot #6	sequence data gene sequence mismatch?	GCA??CGGC??CG?A??GA GTACTCGGCTaCGaACCGA *	Arg2
spot #9	sequence data gene sequence mismatch?	GU?AGGGUGA??UC GTaAGGGTGAGgTC	Thr3
spot #10	sequence data gene sequence mismatch?	GAGGC?CAGGACA GAGGCCCAGGACA	Glu2
spot #12	sequence data gene sequence mismatch?	GAAGGUGGCGGAA...GCUAAGC GAAGGTGGCGGAA...GCTA-GC (*)	Leu1

(a) "?" indicates a base that can not be identified; "-" indicates a base present in the gene sequence but not found in the sequence data. "..." indicates two sequences are separated by an unspecified number of bases.

(b) "-" indicates a base called from the sequence data but not present in the gene sequence. "..." indicates two sequences are separated by an unspecified number of bases. Lower-case letters indicate bases that are modified in mature tRNA (13); these bases may not be properly recognized by the sequence-specific RNases.

(c) "*" indicates a mismatch between the sequence data and the known gene sequence; "(*)" indicates misalignment or a mismatch with a modified base. It is particularly difficult to resolve C/U from the sequence data; four of the five outright mismatches involve these bases.

Table 2. tRNA abundance versus controls (ASDΔ or ASDΔ-SDΔ) in strains making wild-type translating ribosomes (ASDVIII or ASDVIII-SDΔ), specialized non-translating ribosomes (ASDX or ASDX-SDΔ), or specialized translating ribosomes (ASDX-SDX) [a]

		Yamagishi *et al.* (4)		this work				
		λ P_L		λ P_L		*tac*		
		ASDVIII / ASDΔ	ASDX / ASDΔ	ASDVIII / ASDΔ	ASDX / ASDΔ	ASDVIII-SDΔ / ASDΔ-SDΔ	ASDX-SDΔ / ASDΔ-SDΔ	ASDX-SDX / ASDΔ-SDΔ
(I)	Glu2	4.31	6.18	2.53	2.01	2.10	2.85	3.46
(II)	Ile	0.49	0.96	—	—	—	—	—
	Ala1B	0.44	0.78	—	—	—	—	—
	Trp	0.44	0.67	—	—	—	—	—
(III)	Thr3	—	—	0.50	0.58	0.32	0.60	0.72
	Ala2	—	—	0.49	0.56	0.38	0.58	0.66
	Ser3	0.66	1.02	—	—	—	—	—
	Leu1	0.58	0.83	0.57	0.59	0.40	0.57	0.78
	Ser1	0.59	0.97	—	—	—	—	—
	Tyr1+2	0.58	1.06	—	—	—	—	—
	Met(m)	0.58	0.96	—	—	—	—	—
	Asn	0.67	0.83	—	—	—	—	—
	His	0.50	0.89	—	—	—	—	—
	Arg	0.58	0.95	0.61	0.64	0.34	0.54	0.78
	Phe	0.55	0.99	—	—	—	—	—
	Val1	0.53	0.88	—	—	—	—	—
	Gly3	0.57	0.99	—	—	—	—	—
(IV)	spot #1	—	—	0.51	0.59	0.45	0.68	0.86
	spot #2	—	—	0.49	0.54	0.36	0.60	0.75
	spot #5	—	—	0.50	0.52	0.34	0.56	0.77
	spot #7	—	—	0.53	0.52	0.42	0.65	0.80
	spot #8	—	—	0.53	0.61	0.36	0.60	0.72
	spot #11	—	—	0.56	0.66	0.42	0.57	0.66
	spot #13	—	—	0.53	0.63	0.33	0.63	0.76
	average (II-IV) [b]	0.55 ±0.07	0.91 ±0.10	0.53 ±0.04	0.59 ±0.05	0.37 ±0.04	0.60 ±0.04	0.75 ±0.06
	promoter strength [c]	15.0	21.1	8.0	5.7	6.9	9.0	10.9

(a) Strains are identified as follows: ASDΔ = NO3203/P_L-Δ*Sst*, ASD(VIII,X) = NO3203/pASD(VIII,X)-P_L, ASDΔ-SDΔ = JM109/ pRJL1-Δ*Sac*II/pLOU1-Δ*copB*p, ASD(VIII,X)-SDΔ = JM109/ pRJL1-ASD(VIII,X)/pLOU1-Δ*copB*p, ASDX-SDX = JM109/pRJL1-ASDX/pLOU1-SDX.

(b) Values shown for this work are averages from two experiments for the NO3203 strains (one with each medium, see **Materials and Methods**) and two to four experiments for the JM109 strains. tRNAs are grouped according to Yamagishi *et al.* (4): group I are plasmid-encoded tRNAs (Glu2), group II are tRNAs encoded in chromosomal rRNA operons, and group III are tRNAs encoded on the chromosome but not structurally coupled to rRNA operons. In addition, we analyzed several spots that were not unambiguously identified (group IV). Averages are shown ± 1 SD.

(c) Promoter strengths were calculated from the tRNA abundance data as described in the text.

Calculation of plasmid-borne promoter strengths

The amount of $tRNA_2^{Glu}$ synthesized by the control strains is equal to the product of four $tRNA_2^{Glu}$ genes (12) and one arbitrarily-defined transcription unit per gene. The amount synthesized by the experimental strains is equal to the amount of $tRNA_2^{Glu}$ synthesized from the plasmid (P) plus the product of four $tRNA_2^{Glu}$ genes and the measured transcription per gene (the average abundance relative to the control of all tRNAs except $tRNA_2^{Glu}$). The ratio of these values

(experimental/control) is shown in Table 2 as *Glu2*. This equation can be rearranged to solve for the plasmid-borne promoter strength. The resulting values of *P*, in the same arbitrarily-defined transcription units, are also shown in Table 2.

$$Glu2 = \frac{P + (4)(tRNA)}{(4)(1)} \Rightarrow P = (4)(1)(Glu2) - (4)(tRNA) = (4)(Glu2 - tRNA)$$

This measure of transcription (*P*) incorporates both plasmid copy number and intrinsic promoter strength effects, but it is primarily changes in promoter strength we measured. Yamagishi *et al.* (4) reported, and we confirmed, less than 10% variation in plasmid copy number for their strains; results for our strains showed variation of less than 15% (data not shown).

Discussion

To study feedback regulation of rRNA synthesis, we repeated the experiments of Yamagishi *et al.* (4) using their plasmids and host strain and did similar experiments with our plasmids. In every case, substantial repression (45-63%) of chromosomal tRNA synthesis was observed following synthesis of wild-type ribosomes. However, following synthesis of non-translating specialized ribosomes, Yamagishi *et al.* (4) saw less than 10% repression, concluding that there was no feedback regulation; we saw about 40% repression with both their system and ours.

The plasmids and host strain used by Yamagishi *et al.* (4) were obtained from the authors of that paper, and we confirmed the identity of the plasmids by restriction analysis and by sequencing the ASD regions (data not shown). We first used a growth medium slightly different from that used by Yamagishi *et al.* (4), but we then repeated the experiment with the medium they described. The regulation results were essentially the same with the two media.

The different results may be related to the temperature induction. Denaturation of the *cI857* repressor and subsequent transcription from the λP_L promoter may be sensitive to the final temperature achieved and the rate at which the temperature is shifted. These parameters in turn depend on such unspecified factors as the volume of the culture; the size, type, and material of construction of the culture vessel; the type of incubator (air or water); and how temperature is controlled during addition of the radioactive label. We note that the amount of transcription we observed from the plasmid was considerably less than that reported by Yamagishi *et al.* (4) and that we saw much more variability in the Glu2 values than we saw for tRNAs encoded only on the chromosome.

We were initially concerned that, compared to Yamagishi *et al.* (4), we saw less Glu2 overproduction but as much or more regulation. However, if we compare their results to an earlier study from the same lab (2), we find an almost 20% increase in the overproduction of Glu2 accompanied by a 25% increase in chromosomal tRNA synthesis (*i.e.*, less regulation). Our results with their strains are consistent with those of Gourse *et al.* (2) — less Glu2 overproduction and less regulation. In pairwise comparisons of the three studies, Yamagishi *et al.* (4) is inconsistent with both Gourse *et al.* (2) and us, but our results and those of Gourse *et al.* (2) are consistent.

As for our results with *tac*, differences in the plasmid constructions and the host strain may account for the quantitatively different relationship between Glu2 overproduction and feedback

regulation. Gourse *et al.* (2) and Yamagishi *et al.* (4) used identical host strains and plasmids (except for the orientation of the *rrnB* operon in P_L-*rrnB* and pASDVIII-P_L, respectively); the induction times (30 and 15 min) and labeling times (60 and 40 min) were comparable. If such different results can arise in systems so nearly similar, it is perhaps not surprising that our results with a different host strain and plasmids show a quantitatively different relationship between the overproduction of plasmid-borne rRNA and the inhibition of chromosomal rRNA synthesis.

When we added specialized mRNA to our specialized ribosome system, we expected to restore the feedback regulation we assumed would be absent in the non-translating system. While our non-translating system showed substantial regulation, there was markedly more with our wild-type system. We thought that enabling translation on our specialized ribosomes might restore regulation to levels observed following synthesis of wild-type ribosomes, but the repression seen following synthesis of translating specialized ribosomes (25%) was somewhat less than for non-translating specialized ribosomes (40%) and much less than for wild-type ribosomes (63%).

Our results with two different systems suggest that feedback regulation is operating regardless of whether newly synthesized ribosomes are translating. We were unable to reproduce results to the contrary (4). However, the authors of that paper have considerable experience with the specialized ribosome system and the analytical techniques used. In addition, some features of their data are consistent with our results, as described below. Finally, manipulation of intracellular levels of translation initiation factor IF2 seems to support the view that translation initiation is required for feedback regulation (14). The experimental uncertainties mentioned above might account for the different regulation results, but until these differences are resolved, it seems that the relationship of translation to the regulation of rRNA synthesis is still an open question.

Yamagishi *et al.* (4) observed more transcription of the plasmid-borne rRNA operon with the mutant ASD region (no regulation) than with the wild-type ASD region. They speculated that the λ P_L promoter was sensitive to the effector(s) of feedback regulation of rRNA synthesis, although similar plasmids do not contain the presumed target sites for feedback regulation (2). We found the same relationship between regulation and transcription from the plasmid-borne *tac* promoter.

Since there is no reason to expect that either a bacteriophage promoter (λ P_L) or a synthetic hybrid promoter (*tac*) would respond to regulatory signals specific for the synthesis of rRNA, this co-regulation may reflect a general regulatory mechanism. To investigate this phenomenon, the transcription of chromosomal operons and genes structurally and functionally unrelated to rRNA and tRNA synthesis could be examined. Repression of mRNA synthesis from these loci following synthesis of wild-type rRNA from a plasmid would strongly suggest that regulation of rRNA synthesis is part of a cell-wide regulatory process. We note a report of unpublished results showing inhibition of ribosomal protein mRNA synthesis following synthesis of wild-type rRNA (2).

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