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Specialized ribosome system: Preferential translation of a single mRNA species by a subpopulation of mutated ribosomes in *Escherichia coli*

(Shine–Dalgarno sequence/*rrnB* operon)

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ABSTRACT **In *Escherichia coli*, all mRNAs are translated by one pool of functionally identical ribosomes. Here, we describe a system in which a subpopulation of modified ribosomes are directed to a single mutated mRNA species. This was accomplished by changing the Shine–Dalgarno sequence that precedes the heterologous human growth hormone gene from 5′ GGAGG to 5′ CCTCC or 5′ GTGTG. Translation of these modified mRNAs by wild-type ribosomes is very inefficient. When the anti-Shine–Dalgarno region (i.e., the region complementary to the Shine–Dalgarno sequence) at the 3′ end of the gene encoding 16S rRNA (*rrnB*) was altered from 5′ CCTCC to 5′ GGAGG or 5′ CACAC, thus restoring its potential to base-pair with the mutated human growth hormone mRNA, significant expression of this mRNA occurred. Growth hormone synthesis was dependent on induction of the mutated *rrnB* operon. Subsequently, these specialized ribosomes were made spectinomycin-resistant by the introduction of a C→U substitution at position 1192 of the 16S rRNA. Thus, host protein synthesis could be shut off by the addition of spectinomycin and the specificity and efficiency of the specialized ribosomes could be assessed. Since the specialized ribosomes represent a nonessential subpopulation in the cell, this system offers an approach to the study of mutations elsewhere in the 16S-rRNA gene that otherwise would be lethal to the cell.**

The Shine–Dalgarno (SD) sequence (1), which is generally found 8 bases upstream from the start codon in *Escherichia coli* mRNAs (2, 3), is complementary to a region close to the 3′ end of 16S rRNA, hereafter referred to as the anti-Shine–Dalgarno (ASD) sequence. Shine and Dalgarno postulated that the SD sequence in the mRNA interacts with the ASD region of 16S rRNA and that this mRNA·rRNA base-pairing plays an important role in the initiation of protein biosynthesis. A large body of biochemical and genetic evidence supports this view (2). The base-paired complexes can be isolated *in vitro* (4), and mutations in the mRNA altering the complementarity have a profound effect on its translatability (5–11). Replacement of the SD sequence with a synthetic DNA fragment encoding a non-SD sequence essentially abolishes mRNA translation (8, 11).

The ASD sequence is part of the highly conserved 3′ end of the 16S rRNA found in prokaryotes (12). Synthetic oligonucleotide probes can be bound specifically to the ASD region of the 16S rRNA in intact 30S ribosomal subunits, implying that this sequence is not complexed with proteins or RNA (13, 14).

Since one pool of ribosomes presumably translates all mRNAs, it is difficult to obtain and study mutations in the rRNA molecule; mutations introduced via site-directed mutagenesis often appear to be lethal (15).

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In this paper, we describe experiments showing that a mutated ribosomal subpopulation can be made and directed to a single mutated mRNA species. This was accomplished by changing the SD sequence on the mRNA as well as the ASD sequence on the 16S rRNA into entirely different but complementary sequences. This system of specialized ribosomes offers a powerful approach to the analysis of additional mutations in crucial regions of the rRNA molecule that otherwise would be lethal to the cell.

MATERIALS AND METHODS

Plasmid amplification was done in *E. coli* 294 (F^-, *supE44, endA1, thi-1, hsdR4*). Transformation with plasmids containing the P_L promoter of bacteriophage λ was done in *E. coli* K5716 (F′, Δ(*lac–pro*), *supE, traD36, proAB, lacI*^{Q} *Z*ΔM15), which is a λ^+ lysogen derived from JM101. Temperature induction of the specialized ribosomes with λ P_L was done in *E. coli* K5637 (*c*I857, ΔBam *cro*^{27}, *O*am^{29}). Both K5716 and K5637 were constructed by H. I. Miller (Genentech).

Plasmid constructions and blot hybridization analyses of electrophoretically fractionated RNA were done as described (10). $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli (16).

RESULTS

Assembly of the Specialized Ribosome System. In order to assemble a system in which a ribosomal subpopulation would translate a single mRNA species, it was necessary to alter the SD sequence and the ASD sequence in such a way that wild-type ribosomes would not recognize the mutated mRNA and that mutated ribosomes would not recognize the wild-type mRNA. One way in which this goal might be accomplished was to replace the natural SD and ASD sequences by their complements on both the mRNA and the 16S rRNA. Thus, we changed the SD sequence from 5′ GGAGG to 5′ CCUCC and the ASD sequence from 5′ CCUCC to 5′ GGAGG; this is system IX. Since we were concerned that these radical changes might impair ribosomal functions, we also assembled a system that differed less radically from the wild-type system. In system X, the SD sequence was changed from 5′ GGAGG to 5′ GUGUG. The ASD sequence was changed accordingly from 5′ CCUCC to 5′ CACAC. In all systems the gene encoding the mutated mRNA is transcribed constitutively by a promoter derived from the *trp* promoter (10), whereas the wild-type and mutated *rrnB* genes are under control of the λ P_L promoter (17).

Abbreviations: ASD sequence, anti-Shine–Dalgarno sequence; hGH, human growth hormone; PSDR, portable Shine–Dalgarno region; SD sequence, Shine–Dalgarno sequence.

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A detailed description of the construction of the entire specialized ribosome system is given elsewhere (10). The essential features of the system are shown in Fig. 1, and a summary of all the plasmids used for the experiments and their controls is given in Table 1.

Induction of the Mutated *rrnB* Operon. Since the ASD region of the specialized rRNA differs from that of the wild type at the 3′ end, it is possible to distinguish the two 16S rRNA species by using radiolabeled oligodeoxynucleotide probes that are specific for each. Plasmids of the pASD-PSDR-P_L series were transformed into the host K5637, which carries a gene encoding a temperature-sensitive λ repressor, and the accumulation of the mutated 16S rRNA was monitored after induction. Fig. 2 shows that the synthetic oligonucleotide probes (their sequences are given in the figure legend) that are complementary to either ASD IX or X do not hybridize to wild-type 16S rRNA (i.e., to rRNA from cells lacking the specialized system). The level of the mutated 16S rRNA increases rapidly and reaches a maximum after about 1 hr of induction (quantitation data not shown). The size of the mutated 16S rRNA molecules is identical to that of wild type, suggesting that the changes in the ASD sequence do not have a major effect on the processing of the rRNA precursor.

Protein Synthesis by the Specialized Ribosomes. Fig. 3 shows the time course of hGH accumulation after induction of the specialized ribosome systems. Note that the hGH gene is transcribed constitutively in all five systems. A minute amount of hGH is detected when specialized mRNA is made in the absence of specialized ribosomes (as in the control systems phGH-PSDRIX and -X). The hGH made here is probably due to the inefficient translation of the specialized mRNAs by the wild-type ribosomes. A low level of hGH is also synthesized in the specialized systems IX and X prior to induction. However, upon induction of P_L, the hGH level gradually increases. After 3 hr of induction, the hGH level in system X is equal to that in the wild-type system. The overall efficiency of system X is 2- to 3-fold higher than that of system IX. The reasons for this difference in efficiency are not clear. The base composition of the SD sequence and ASD region may affect the rate of translation initiation or the rate of elongation differently in the two systems. The hGH-accumulation data (Fig. 3), along with the RNA data (Fig. 2), show that the mutated 16S-rRNA gene is indeed transcribed and that at least some of the mutated rRNA molecules are processed and assembled into functional ribosomes.

$NaDodSO_4$/polyacrylamide gel electrophoresis of total cellular protein extracted before or after induction of cells containing system IX or X reveals the appearance of a protein of 22.5 kDa in both specialized systems [compare lanes 2 and 3 (30°C) with 7 and 8 (37°C) in Fig. 4]. This protein has the same electrophoretic mobility as hGH, and immunoblot analysis confirmed that this protein band is indeed hGH (data not shown). The hGH level found in system X is again greater than that in system IX and is comparable to that found in

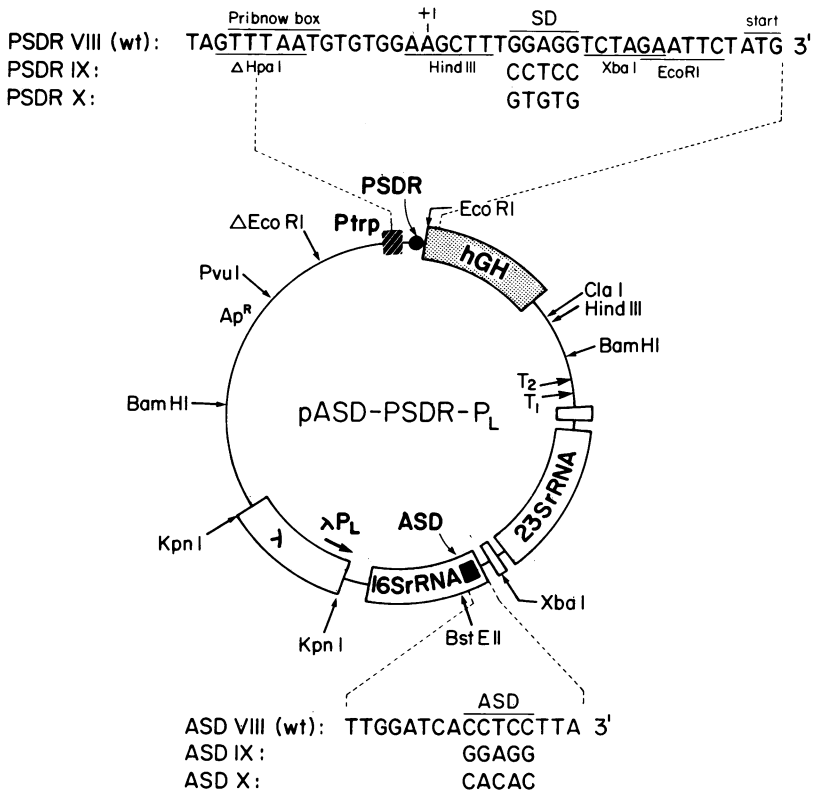

FIG. 1. The plasmid-borne specialized ribosome system. In this system the human growth hormone (hGH) gene is transcribed under the control of a constitutive mutant *trp* promoter (Ptrp) (10) and the natural *trp* leader SD sequence (5′ AAGG) was replaced by the sequence 5′ GGAGG in system VIII by using a double-stranded synthetic DNA fragment (10). The sequence of the modified promoter and "portable" SD region (PSDR) in system VIII ("wild-type," wt) is shown, along with the ATG translation start codon; restriction endonuclease recognition sites are underlined. The untranslated region of the hGH gene in systems IX and X is the same as in system VIII except for the SD region, as shown. The plasmid-borne *rrnB* operon is under λ P_L control and is temperature-inducible due to the presence of the λ *c*I857 repressor in the λ-lysogenic host. The sequence of the 3′ end of the 16S-rRNA gene is shown for ASD VIII, along with the mutated ASD regions in systems IX and X (see ref. 10 for details). Thus, the final pASD-PSDR-P_L plasmids encode hGH mRNA with an SD sequence that is complementary to the ASD sequence of the mutated *rrnB* operon on the same plasmid. Plasmids either lacking the hGH gene [pASDVIII (IX and X)-P_L] or containing the hGH gene with a wild-type SD sequence and a mutated ASD region in the specialized ribosomes (pASDIX-PSDRVIII-P_L and pASDX-PSDRVIII-P_L) were constructed as experimental controls (see Table 1). Introduction of any of these plasmids into the cells is not lethal. T_1 and T_2 are transcription termination sites. Ap^R represents the gene that confers ampicillin resistance.

Table 1. Summary of the plasmids and systems used

Plasmid	16S-rRNA ASD sequence (5′ to 3′)	hGH SD sequence (5′ to 3′)	ASD/SD duplex
phGH-PSDRVIII	*rrnB* not present on plasmid	GGAGG	—
phGH-PSDRIX	*rrnB* not present on plasmid	CCUCC	—
phGH-PSDRX	*rrnB* not present on plasmid	GUGUG	—
pASDVIII-P_L	CCUCC (wild type)	Gene not present on plasmid	—
pASDIX-P_L	GGAGG	Gene not present on plasmid	—
pASDX-P_L	CACAC	Gene not present on plasmid	—
pASDVIII-PSDRVIII-P_L (system VIII)	CCUCC	GGAGG	3′ CCUCC 5′ GGAGG
pASDIX-PSDRI-P_L (system IX)	GGAGG	CCUCC	3′ GGAGG 5′ CCUCC
pASDX-PSDRX-P_L (system X)	CACAC	GUGUG	3′ CACAC 5′ GUGUG
pASDIX-PSDRVIII (system IXc)	GGAGG	GGAGG	3′ GGAGG 5′ GGAGG
pASDX-PSDRVIII (system Xc)	CACAC	GGAGG	3′ CACAC 5′ GGAGG
pASDVIII-PSDRVIII-P_L-Spc^r*			
pASDIX-PSDRIX-P_L-Spc^r†			
pASDX-PSDRX-P_L-Spc^r‡			

*Spectinomycin-resistant derivative of system VIII.
†Spectinomycin-resistant derivative of system IX.
‡Spectinomycin-resistant derivative of system X.

wild-type cells (compare lanes 6 and 8 in Fig. 4). These data are in general agreement with the kinetic data shown in Fig. 3.

In addition to the hGH band, a novel protein of 50 kDa appears in system IX (lane 7) and a protein of 19 kDa in system X (lane 8). Apparently, each kind of specialized ribosome recognizes a sequence in front of a long open reading frame that is not recognized as a binding site for wild-type ribosomes. Immunoblot analysis showed that the 50-kDa protein is not antigenically related to hGH (data not shown) and therefore is not a read-through translation product of the hGH mRNA. However, the 19-kDa protein is immunologically related to hGH. However, the 19-kDa

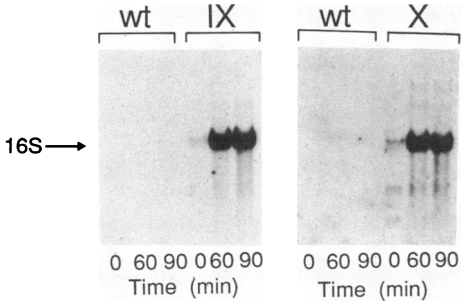

FIG. 2. Blot hybridization analysis of electrophoretically fractionated RNA, showing the induction of *rrnB* operons bearing a mutated ASD region. Cells were grown at 30°C for 2 hr, transferred to 42°C (time zero) for 15 min, and then incubated at 37°C for the remaining time. Total RNA was isolated and analyzed at the various times after induction as described (10). In the left two panels (wild-type cells and system IX), the hybridization probe specific for ASD IX was used; in the right two panels, the probe for ASD X was used. The sequence of the ASD IX probe is 5′ TCTTTAAGGTAACCTCCTGATCCAACCGC and that for ASDX is 5′ TCTTTAAGGTAAGTGTGTGATCCAACCGC.

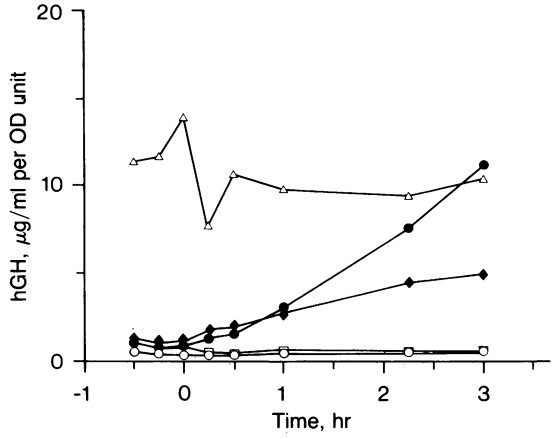

FIG. 3. hGH accumulation in cells with specialized ribosomes. Extraction and assay of hGH were done as described (10). △, Wild-type system VIII; ◆, specialized system IX; ●, specialized system X; ○, control system phGH-PSDRIX; and □, control system phGH-PSDRX. Both control systems contain specialized mRNA but lack specialized ribosomes. Values are normalized to cell culture density (OD unit).

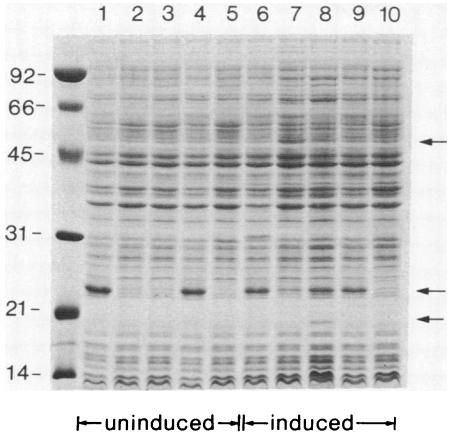

FIG. 4. Protein profile of cells containing the specialized system, before and after induction of the specialized system. For induction, cells were grown at 30°C for 2 hr, then shifted to a 42°C bath for 15 min, and finally transferred to 37°C for 3 hr. Cells were harvested and the protein profile was analyzed by $NaDodSO_4$/12.5% polyacrylamide gel electrophoresis; proteins were visualized by Coomassie blue stain. Lane 1 represents the wild-type system, lanes 2 and 3 the specialized systems IX and X, and lanes 4 and 5 represent the control systems phGH-PSDRVIII and pASDVIII-P_L, all at 30°C. Lanes 6–10 represent the same systems in the same order but at 37°C. The position of hGH (22.5 kDa) is indicated by the middle arrow. The upper and lower arrows indicate the novel bands (50 kDa and 19 kDa) that appear in systems IX and X, respectively, at 37°C. Leftmost lane shows standard proteins, whose molecular masses are given in kDa.

protein is immunologically related to hGH. Lane 4 and lane 9 (Fig. 4) show the protein profile in cells (at 30°C and 37°C, respectively) that make a wild-type hGH mRNA constitutively but lack specialized ribosomes. The hGH band is present at both temperatures, as expected (the hGH mRNA is translated by wild-type ribosomes). However, the novel proteins of 50 kDa and 19 kDa are absent at 37°C, indicating that these novel proteins as found in systems IX and X are not heat shock proteins. Lanes 5 and 10 show the protein profiles of negative control strains containing plasmids encoding a wild-type plasmid-borne *rrnB* operon without the hGH gene.

Specificity and Efficiency of the Specialized Ribosome System. To investigate the relative efficiency and mRNA specificity of the specialized ribosomes in translating endogenous wild-type messengers, it is essential to inactivate the wild-type ribosomes derived from chromosomal *rrn* operons. Hence, the spectinomycin-resistance mutation (18) was introduced into the rRNA of the specialized ribosomes. The specialized ribosomes were made spectinomycin-resistant by introducing a C→U substitution at position 1192 of the 16S rRNA molecule by site-directed mutagenesis (10). The resulting plasmids pASDVIII (IX and X)-PSDRVIII (IX and X)-P_L-Spc^r were used to transform *E. coli* K5637, which contains the temperature-sensitive λ repressor. After 2 hr of induction, spectinomycin was added to inactivate the wild-type ribosomes; 5 min later, [^{35}S]methionine was added to label the proteins translated by the spectinomycin-resistant specialized ribosomes. The radioactive protein profile was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by autoradiography (Fig. 5). The protein profile of cells containing system VIII-Spc^r (lane 1) is, as expected, similar to that of cells grown in the absence of the antibiotic (lane 10). In the protein profiles of cells having the specialized systems IX-Spc^r and X-Spc^r (lanes 2 and 3, respectively), hGH is the most prominent band (25–30% of total newly synthesized proteins). Comparison of the total amount of labeled hGH produced in both systems confirms previous results showing that the overall efficiency of system X is 2- to 3-fold higher than that of system IX. Lanes 6–8 show the

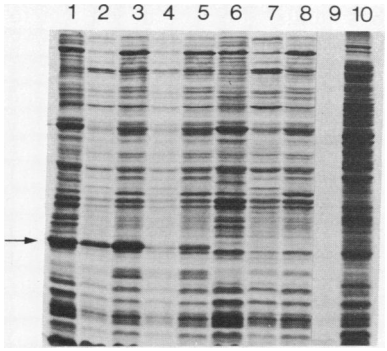

FIG. 5. [^{35}S]Methionine incorporation into proteins synthesized by spectinomycin-resistant specialized ribosomes in the presence of spectinomycin. Protein profiles from *E. coli* K5637 (*c*I857) cells containing the various plasmids are shown. Cells were grown at 30°C for 2 hr in M9 medium without Casamino acids. After 2 hr of induction at 37°C, spectinomycin (GIBCO) was added (0.5 mg/ml); 5 min later, [^{35}S]methionine (760 μCi/μM; 1 Ci = 37 GBq) was added. Proteins were extracted after 30 min of incubation and analyzed by $NaDodSO_4$/12.5% polyacrylamide gel electrophoresis. An autoradiograph is shown. Arrow indicates hGH band. Lanes 1–3: the three Spc^r systems (VIII, IX, and X) in the presence of spectinomycin. Lanes 4 and 5: controls showing [^{35}S]methionine incorporation in cells with pASDIX-PSDRVIII-P_L-Spc^r and pASDX-PSDRVIII-P_L-Spc^r, respectively. Lanes 6–8: cells containing pASDVIII (IX,X)-P_L-Spc^r; these plasmids lack the hGH gene but are otherwise like the systems VIII, IX, and X, respectively. Lane 9: background incorporation in the cells containing system VIII (lacking the spectinomycin-resistance mutation) but in the presence of the antibiotic. Lane 10: same as lane 9, but in the absence of the antibiotic. Extract from the same amount of cells was loaded in each lane (i.e., 0.15 OD_{550} equivalent per lane). The relative hGH levels (determined by a laser scan of a lighter exposure) as percentage of total radioactivity loaded are as follows: lane 1, 11%; lane 2, 25%; lane 3, 28%; lane 4, 6%; lane 5, 6%.

protein profiles of cells containing the same three systems (pASDVIII, -IX-, and X-Spc^r) but lacking the hGH gene. Lane 9 reflects the background protein synthesis by wild-type ribosomes (spectinomycin-sensitive) derived from plasmid and chromosome in the presence of the antibiotic. At this exposure time, hardly any incorporation is visible. Therefore, the radioactive bands present in the background of lanes 2 and 3 reflect the incorporation in proteins that are the products of wild-type mRNAs translated by the spectinomycin-resistant specialized ribosomes. All these protein species are less abundant than hGH, suggesting that the specialized hGH mRNA is preferentially translated by the specialized ribosomes. However, since the hGH gene is present on a multicopy plasmid and is transcribed by a strong constitutive promoter, the high hGH level observed might merely reflect a high hGH mRNA concentration relative to other cellular mRNAs rather than differences in specificity of the mutated ribosomes for the specialized hGH messengers. To resolve this, the control plasmids (pASDIX-PSDRVIII-P_L-Spc^r and pASDX-PSDRVIII-P_L-Spc^r) were constructed, from which wild-type hGH mRNA is made at equally high levels in cells containing drug-resistant specialized ribosomes. Lanes 4 and 5 show that the hGH mRNA is translated in both cases; however, the relative hGH level is one-fourth to one-fifth that in systems IX and X (see legend to Fig. 5). The absolute hGH level in cells containing pASDX-PSDRVIII-P_L-Spc^r (lane 5) is greater than that in cells containing pASDIX-PSDRVIII-P_L-Spc^r (lane 4), which presumably reflects the higher number of matched bases in the ASD X/SD VIII duplex (see Table 1). All the hGH made here (lanes 4 and 5) originates from specialized ribosomes translating wild-type hGH mRNA, since the wild-type ribosomes are inactivated. This result shows that the predominance of the hGH band in

systems IX-Spc^r and X-Spc^r is indeed due to preferential translation of the specialized hGH mRNAs by the specialized ribosomes. Comparison of the background protein profiles in systems IX-Spc^r and X-Spc^r (lanes 2 and 3 and also lanes 4 and 5) shows that there are several differences in the relative intensities of the background protein bands. This indicates that the relative efficiencies at which both kinds of drug-resistant specialized ribosomes recognize the various endogenous messengers differ substantially. It would be interesting to examine the ribosome recognition sequence of the mRNAs encoding these background proteins. This issue is addressed in more detail by Jacob *et al.* in ref. 19.

DISCUSSION

We have described a system in which one modified mRNA species can be preferentially translated by a modified subpopulation of ribosomes. Our results show that the natural sequences used by the natural translation initiation system in *E. coli* can be replaced by unrelated sequences provided that complementarity is ensured. We do not know whether the initiation frequency of the specialized ribosomes on the specialized mRNA is the same as the initiation frequency on the same mRNA with a wild-type SD/ASD complement. This question cannot yet be answered using the present *in vivo* system, since it is not known whether all specialized rRNA is assembled into active ribosomal particles. Since significant quantities of hGH are made in our system, at least a fraction of the specialized rRNA pool must be packaged into active ribosomes. The size of this fraction can be estimated. After 3 hr of induction, the hGH production of the specialized system X is the same as that of the wild-type system. Since hGH accounts for about 10% of total protein, about 10% of all ribosomes must be engaged in hGH synthesis in the wild-type system. If we assume that the initiation frequency and the elongation rate of the specialized ribosomes is equal or less than that of wild-type ribosomes, then we can conclude that the number of active specialized ribosomes is at least equal to 10% of the number of ribosomes present in the cell.

Regardless of the efficiency, we believe that the system described here offers an approach in studying additional mutations anywhere else in the rRNA molecule. Since there are seven *rrn* operons encoding essentially identical molecules, mutations in one of the wild-type *rrn* operons either are dominant and lethal to the cell or are without phenotype. With the specialized system, it should be possible to introduce mutations anywhere in the specialized rRNA and to study their effects on the translation of one particular specialized mRNA. Following this approach, we have introduced several mutations in the highly conserved C^{1400} region of the 16S rRNA molecule. No effect on the growth rate of the cells containing such a mutation on a plasmid-borne specialized *rrn* operon was observed, as expected, whereas translation of the specialized mRNA was essentially abolished (unpublished observation). This system is also of interest in studies of the role of (free) ribosomes on the regulation of rRNA synthesis. Recently, it was found that specialized ribosomes (of system X) that are inefficient in translating endogenous mRNAs are unable to cause feedback regulation of rRNA synthesis (M. Yamagishi, H.A.deB., and M. Nomura, unpublished results).

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