The Highly Efficient Translation Initiation Region from the Escherichia coli rpsA Gene Lacks a Shine-Dalgarno Element

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In both prokaryotes and eukaryotes, mRNAs are translated with different efficiencies depending upon their sequence and structure. In Escherichia coli, the mRNA region important for translation initiation (translation initiation region [TIR]) usually spans only a few tens of nucleotides bracketing the translation start (11). Besides the initiation codon itself (most commonly AUG [83%] and less frequently GUG [14%] or UUG [3%] [25]), the TIR generally contains a Shine-Dalgarno sequence (SD) element, and it can fold into three successive hairpins (I, II, and III) that are essential for high translational activity. Two conserved GGA trinucleotides, present in the loops of hairpins I and II, have been proposed to form a discontinuous SD. Here, we have tested this hypothesis with the "specialized ribosome" approach. Depending upon the constructs used, translation initiation was decreased three- to sevenfold upon changing the conserved GGA to CCU. However, although chemical probing showed that the mutated trinucleotides were accessible, no restoration was observed when the ribosome anti-SR was symmetrically changed from CUCUC to GGAGG. When the same change was introduced in the SD from a conventional TIR as a control, activity was stimulated. This result suggests that the GGA trinucleotides do not form a discontinuous SD. Others hypotheses that may account for their role are discussed. Curiously, we also find that, when expressed at moderate level (30 to 40% of total ribosomes), specialized ribosomes are only twofold disadvantaged over normal ribosomes for the translation of bulk cellular mRNAs. These findings suggest that, under these conditions, the SD–anti-SR interaction plays a significant but not essential role for the synthesis of bulk cellular proteins.

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In both prokaryotes and eukaryotes, mRNAs are translated with different efficiencies depending upon their sequence and structure. In Escherichia coli, the mRNA region important for translation initiation (translation initiation region [TIR]) usually spans only a few tens of nucleotides bracketing the translation start (11). Besides the initiation codon itself (most commonly AUG [83%] and less frequently GUG [14%] or UUG [3%] [25]), the TIR generally contains a Shine-Dalgarno sequence (SD) element, and it can fold into three successive hairpins (I, II, and III) that are essential for high translational activity. Two conserved GGA trinucleotides, present in the loops of hairpins I and II, have been proposed to form a discontinuous SD. Here, we have tested this hypothesis with the "specialized ribosome" approach. Depending upon the constructs used, translation initiation was decreased three- to sevenfold upon changing the conserved GGA to CCU. However, although chemical probing showed that the mutated trinucleotides were accessible, no restoration was observed when the ribosome anti-SR was symmetrically changed from CUCUC to GGAGG. When the same change was introduced in the SD from a conventional TIR as a control, activity was stimulated. This result suggests that the GGA trinucleotides do not form a discontinuous SD. Others hypotheses that may account for their role are discussed. Curiously, we also find that, when expressed at moderate level (30 to 40% of total ribosomes), specialized ribosomes are only twofold disadvantaged over normal ribosomes for the translation of bulk cellular mRNAs. These findings suggest that, under these conditions, the SD–anti-SR interaction plays a significant but not essential role for the synthesis of bulk cellular proteins.
Hairpins I and II are very stable, whereas hairpin III, which contains the initiator codon in the apical loop and the vestigial SD in the stem, is less so (Fig. 1). This phylogenetically conserved structure is required for the high translational activity of the TIR in *E. coli*, as well as for the autoregulation exerted by the ribosomal protein S1 on its own translation (3, 41). Presumably, the specific folding of the *rpsA* TIR generates an optimal spatial arrangement of sequence elements that interact with the 30S ribosomal subunit (3). Two conserved GGA motifs present in apical loops of hairpins I and II (Fig. 1) presumably stand among such elements. These motifs, which are individually necessary for high translation activity, are separated by 39 nt from each other and by 79 and 40 nt, respectively, from the initiation codon (Fig. 1) (3). One intriguing possibility is that, in spite of this large separation, the structure of the TIR brings them close enough to each other and to the initiation codon to form a discontinuous SD. Overall this discontinuous SD (GGAGGGA) would show a much higher complementarity to the anti-SD than the vestigial SD-like element (Fig. 2B). Here, we have tested this hypothesis with the same specialized ribosome approach previously used for establishing the canonical SD-ASD interaction (17, 20, 23). Our findings do not support the discontinuous SD hypothesis. The effect of the conserved GGA on translation initiation must therefore be explained by other mechanisms, and plausible models are discussed. Interestingly, our data also
suggest that, under the conditions used here, specialized ribosomes are only moderately discriminated against for the translation of bulk cellular mRNA. The significance of this finding is discussed.

MATERIALS AND METHODS

Bacterial strains. The Lac+ strain HfrG6Δ12 (11) was used for the construction of all chromosomal rpsA-lacz and galF-lacZ fusions. This strain carries a short chromosomal deletion of the promoter and the RBS of the lacZ gene (nucleotides −52 to +20 [all numbering refers to the first base of the initiation codon]). Strains DH5α (supE44 hsdR17 recA1 endA1 gyrA46 thi-1 relA1 lac Ile ProAB Tn10 (Tetr)) or XL1-Blue (supE44 hsdR17 recA1 endA1 gyrA46 thi-1 relA1 lac F'). [proAB- lacF3 lacZDM15 Tn10 (Tetr)] were used for plasmid propagation.

Constructions of rpsA-lacz and galF-lacZ translational fusions. Plasmid pES191 is a pEMBL46 derivative (11) that carries a translational fusion between the wild-type rpsA TIR sequence (nucleotides −91 to +57) and the 5′ coding sequence of the lacZ gene (nucleotides +22 to +204) (3). This plasmid was used to introduce deletion and simple or multiple mutations in the rpsA TIR by using either DpnI mutagenesis (Stratagene) or PCR fusion mutagenesis with appropriate oligonucleotides. Mutations were introduced by successive mutagenesis of various pES191 derivatives. All resulting plasmids were checked by sequencing and then used to transfer the mutated rpsA-lacZ fusion onto the chromosome of HfrG6Δ12 by homologous recombination (11). The resulting HfrG6Δ12 derivatives were checked by sequencing of PCR-amplified chromosomal fragments encompassing the rpsA-lacZ fusion regions.

Similarly, plasmid pΔgalE-lacZ is a pEMBL46 derivative that carries a translational fusion between the wild-type galF RBS sequence (nucleotides −35 to +28) and the 5′ coding sequence of the lacZ gene (43). Long-range DpnI PCR over PCR-generated fragments encompassing the galF-lacZ fusion regions. The C1192T mutation was used to generate chromosomal fragments encompassing the TIRs of other genes (2, 38) by using primer extension (38) (Fig. 3A and B). The strain constructed carries a C1192T mutation that confers spectinomycin resistance (38). These modified plasmids were transferred onto the chromosome of HfrG6Δ12 as described above.

Specialized ribosome system. The specialized ribosome system is carried by the kanamycin-resistant plasmids pOFX503 and pOFX504. The p15A derivatives (Kam) contain arabinoside-inducible mbb operons encoding 16S rRNA with wild-type (caCCUCCuua) or mutated (caGGAGGuua) anti-SD, respectively. Plasmids pOFX503 and pOFX504 were further modified by PCR fusion mutagenesis to introduce a C52 to T52 (all numberings refer to the first base of the initiation AUG) de -

RESULTS

Overall design of the experimental system. Throughout this work, the translational activity of wild-type or mutated TIRs was quantified by monitoring the expression of β-galactosidase from suitable chromosomal fusions, as previously described (2, 43). Briefly, the region of the chromosome carrying the genuine lacZ TIR (from position −52 to +20 with respect to the first base of the initiator AUG) has been replaced by DNA fragments encompassing the TIRs of other genes (galE and rpsC in this work) so that β-galactosidase synthesis is driven at the transcription level by the lac promoter and at the translation level by the inserted TIR (Fig. 2A; see Materials and Methods).

The specialized ribosome system consists of two p15A-based plasmids (10 to 12 copies/cell) carrying the mbb operon under each assay of ASDWT or ASDmut ribosomes, cultures were treated for at least 2 h with arabinose (0.2%), and then IPTG (0.5 mM) was added to induce the lac operon. Incubation was continued for an additional 30 min before harvesting of cells.

2D gel electrophoresis. Cells were grown in 20 ml of LB supplemented with arabinose (0.2%) and kanamycin (50 µg/ml) and harvested at an OD600 of 0.5. Pellets were treated with Bugbuster (Novagen) supplemented with Complete EDTA-free protein inhibitors (Roche), 1 unit/ml benzamidine, and 400 µg/ml lysozyme, according to the manufacturer’s protocol (Novagen). Soluble proteins (500 µg) were resuspended in 400 µl of solubilization buffer (8 M urea, 2% 3-[3-cholamidopropyl]-dimethyammonio)-1-propanesulfonate (CHAPS), 40 mM dithiothreitol) and analyzed by two-dimensional (2D) gel electrophoresis (first dimension, Immobiline DryStrip, pH 4 to 7 [Amersham]; second dimension, 10% acrylamide–sodium dodeyl sulfate gel). Gels were stained with Coomassie blue G250 and scanned on a GS-800 imaging densitometer (Bio-Rad). Individual spots were quantified with PDQuest software for 2D analysis (Bio-Rad).

In vivo protein labeling. Cells were grown in M63 minimal medium (28) supplemented with vitamin B1 (0.05%), histidine (0.01%), glycerol (0.9%), and kanamycin (50 µg/ml) to an OD600 of 0.15 to 0.2. Cultures were then supplemented with arabinose (0.2%) and, after 3 hours, with spectinomycin (500 µg/ml). Aliquots (0.5 ml) were labeled with 50 µCi of [35S]Met (Prom; Amersham) either immediately or 30 min after spectinomycin addition. Labeling was stopped after 1 min with excess cold Met and Cys. Cells were harvested, lysed by sonication, and analyzed on a 10% NuPage gel (Invitrogen).

Leak(lII) acetylase probing of rps4 mRNA. Probing was performed on 157-nucleotide-long transcripts encompassing the rps4 operon. The C1192T mutation was used to generate chromosomal fragments encompassing the rps4 operon. Incubation was continued for an additional 30 min before harvesting of cells. For this assay of ASDWT or ASDmut ribosomes, cultures were treated for at least 2 h with arabinose (0.2%), and then IPTG (0.5 mM) was added to induce the lac operon. Incubation was continued for an additional 30 min before harvesting of cells.
A control of an arabinose-inducible promoter (Fig. 2A). In one of them (hereafter referred to as pASDWT), the anti-SD is wild type, whereas in the other (pASDmut) it has been changed from CCUCC to GGAGG (Fig. 2A). Ribosomes containing the modified 16S rRNA should recognize the altered SD CCUCC. In order to tag plasmid-encoded rRNA within the ribosome population, a C→T transition was introduced in both plasmids at position 1192 of 16S rRNA (see Materials and Methods). This mutation, which also confers spectinomycin resistance, can be used to differentiate plasmid-encoded from chromosome-encoded 16S rRNA by primer extension (38) (Fig. 3A). For simplicity, “ASD WT” and “ASDmut” are used hereafter to designate rRNAs from plasmids pASDWT and pASDmut, respectively, or more generally for ribosomes or cells that contain these rRNAs. In contrast, “cellular” is used to designate chromosome-encoded 16S rRNA or the ribosomes that contain it.

Table 1. Distribution of plasmid-encoded 16S rRNA in the 30S subunit, the 70S particle, and polysomes

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>% Plasmid-encoded 16S rRNA (mean ± SD)†</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>pASDWT</td>
<td>38 ± 3</td>
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<tr>
<td>pASDmut</td>
<td>35 ± 4</td>
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†The percentage of plasmid-encoded 16S rRNA is taken as the ratio of the intensity of the +39 signal to the summed intensities of the +19 and +39 signals (see Fig. 3B). Cells were grown in LB medium and induced with arabinose for 3 h.

Specialized ribosomes can translate bulk cellular mRNA. To monitor the effect of specialized ribosomes on cell physiology, cells containing plasmid pASDWT or pASDmut were
grown in LB medium, and arabinose was added in log phase to induce expression of the plasmidic rmb operon. After 2 h of induction, this expression, quantified by primer extension, reached a plateau corresponding to 35 to 38% of total 16S rRNA (Table 1); the value for ASDmut RNA is consistently slightly lower than for ASDWT, perhaps indicating some instability of the former rRNA in vivo. Importantly, induction did not compromise steady-state growth. With ASDWT rRNA the growth rate was unaffected, whereas with ASDmut it was depressed by only ca. 10%, indicating that protein synthesis is not severely impaired (data not shown). To assess whether ASDmut rRNA is incorporated into active ribosomes, its distribution into the 30S subunit, the 70S particle, and polysomes was determined. To this end, cell extracts were fractionated on sucrose gradients, and the 16S rRNA population from individual fractions was analyzed (Fig. 3A and B). As expected, the ASDWT 16S RNA was efficiently incorporated in polysomes; however, quantitatively, its relative abundance in polysomes was only 80% of that in 30S subunits (i.e., ca. 30/38) (Table 1), possibly indicating that, functionally, the C1192T mutation is not completely neutral (10, 35). Surprisingly, the ASDmut 16S rRNA was also well represented in polysomes (Fig. 3B), with a proportion that reached ca. 45% of that in 30S subunits (i.e., 15/33) (Table 1). Obviously, ribosomes bearing ASDmut are not strongly counterselected for translation, even though the cells contain no engineered mRNA with a complementary SD.

To determine whether ASDmut ribosomes participate normally in cellular translation, protein extracts from ASDWT or ASDmut cells were compared by 2D gel electrophoresis (Fig. 3C). Quantification of 178 spots from Coomassie blue-stained gels showed that the two protein profiles were similar, with only 10% of the spots differing more than twofold in intensity between the two strains (Fig. 3C). The scarcity of ASDmut, specific spots on the 2D gels suggests that ASDmut ribosomes do not concentrate on a few specific mRNAs but participate in the translation of many, and perhaps most, well-translated cellular mRNAs.

The above conclusion seems at odd with previous work showing that ribosomes carrying mutated ASDs, including the ASDmut sequence used here (system IX in reference 17) cannot translate cellular mRNAs normally (17, 20). A distinctive feature of these former studies compared to the present one is that the proportion of mutated versus cellular ribosomes was much higher. To test whether this higher proportion might explain the difference, we exploited the fact that in the presence of spectinomycin, cellular ribosomes are inactivated so that ASDWT or ASDmut ribosomes now constitute 100% of the ribosomes active in the cell. With the spectinomycin concentrations required to inhibit cellular ribosomes almost completely (i.e., 500 μg/ml), neither ASDWT nor ASDmut cells could achieve steady-state growth, perhaps because the concentration of spectinomycin-resistant ribosomes in the cell is insufficient. Nevertheless, protein synthesis in the two strains could be compared by adding the drug to growing cells and then pulse-labeling them with [35S]methionine (see Materials and Methods). As expected, with ASDWT cells, protein synthesis was reduced but still observable in the presence of the drug. Interestingly, the same hold true also for ASDmut cells (Fig. 3D). Comparison of the two protein profiles shows a few individual species that are synthesized mainly or exclusively either by the ASDWT or the ASDmut ribosomes, together with a background that appears similar in the two cases (Fig. 3D). Altogether, quantification showed that, in the presence of spectinomycin, 35S incorporation in ASDmut cells was quite significant (ca. 50%) compared to that in ASDWT cells, confirming that ASDmut ribosomes can translate bulk cellular mRNAs.

In the past, specialized ribosomes have been assayed either under conditions illustrated above, i.e., moderate expression allowing steady-state growth (1, 23, 31), or under conditions of high expression and/or selective inactivation of cellular ribosomes, allowing only transient studies (5, 15, 17, 20). The former conditions have been preferred here, since steady-state conditions appear more physiologically relevant. Since ASDmut ribosomes are then assayed in the presence of excess cellular ribosomes, their preferential utilization by a given TIR is detected as an increase in TIR activity over a nonzero background due to cellular ribosomes. Previously, this background has been taken as the activity of the TIR prior to induction of the specialized system (23, 31). For greater consistency, the activity of the TIR in the presence of ASDWT ribosomes is used here for this purpose.

ASDmut ribosomes preferentially translate an mRNA with a complementary SD. The specificity of the ASDmut ribosomes was first evaluated with lacZ chromosomal fusions carrying the TIR from the galE gene. This particular TIR was chosen because its activity is stringently dependent upon the SD-ASD interaction (43). The galE SD, GGAG(C), was mutated to CCUC, and strains carrying the wild-type or mutated galE TIR were then transformed with plasmid pASDWT or pASDmut. After at least 2 hours of arabinose induction so that the concentration of plasmid-derived ribosomes reached a plateau, the lac operon was induced with IPTG and β-galactosidase expression was measured.

The activity of the wild-type galE TIR was high and was nearly identical whether ASDWT or ASDmut ribosomes were present (Fig. 4A). Concerning the mutant TIR, its activity was very low in the presence of ASDWT ribosomes (ca. 1.5% of the wild-type value), consistent with the known stringency for SD-ASD interaction in this case. In the presence of ASDmut ribosomes, this low activity was stimulated ~6-fold. Yet, activity still amounted to only 10% of the activity of the wild-type TIR. It is possible that compared with the wild-type TIR, the mutant TIR adopts a structure less favorable for ribosome binding. Alternatively, specialized ribosomes may be less efficient than the wild-type ribosomes for recognizing their cognate SD; one obvious reason is their lower concentration in the cytosol, which may not be enough to saturate the TIR. In any case, this experiment shows that the ASDmut ribosomes can indeed recognize the modified SD, CCUC.

The conserved GGA motifs in apical loops I and II of the rpsA TIR do not act as a discontinuous SD element. To test whether the conserved GGA motifs in the apical loops of hairpins I and II of the rpsA TIR constitute a discontinuous SD element, we simultaneously mutated them to CCU (loop I+IImut,AUG in Fig. 1). Interestingly, this double replacement did not reduce the expression of the rpsA-lacZ fusion more than a single GGA→CCU replacement in loop I (result not shown) or than individual GG→AU replacements in either loop (3). The fact that alterations in either conserved
A fairly efficiently; its activity in the presence of ASD WT rpsA wild-type above. Like for the wild-type //H11001 IImut) (lower panel) in loops I and II, together with the motifs (loopI WT) (upper panel) or the mutant CCU //H11001 ASDmut ribosomes. -nitrophenyl- in independent assays. (A) of total protein. The values shown are averages from at least three stringently dependent upon the presence of the GGA ele-

FIG. 4. Histograms showing β-galactosidase activities from the indicated TIR-lacZ translational fusions in the presence of ASD WT or ASD mut ribosomes. β-Galactosidase units correspond to nanomoles of o-nitrophenyl-β-D-galactopyranoside hydrolyzed per min and per mg of total protein. The values shown are averages from at least three independent assays. (A) β-Galactosidase expression from the galE WT and galECCC TIRs. (B) β-Galactosidase expression from various mutants of the rpsA TIR. The different TIRs contain either the wild-type GGA motifs (loopI+II WT) (upper panel) or the mutant CCU motifs (loopI+II mut) (lower panel) in loops I and II, together with the indicated additional mutations (see Fig. 1). Note the different scales of individual histograms. Error bars indicate standard deviations.

GGA motif, or in both of them, affect translation to the same extent supports the view that these motifs work together for optimal TIR activity.

Plasmid pASD WT or pASD mut was then introduced into the strains carrying either the wild-type or the (loopI+II mut,AUG) TIR, and the activities of the TIRs were evaluated as described above. Like for the wild-type galE TIR, the activity of the wild-type rpsA TIR was not significantly different in the presence of either type of ribosome (Fig. 4B). In contrast, the activity of the (loopI+II WT,AUG) TIR was higher in the presence of the ASD mut ribosomes, showing that the latter use the mutated TIR more efficiently than the ASD WT ribosomes. Yet the effect (1.3-fold) was far more modest than that with the galE TIR (6-fold). An obvious reason for this smaller effect was that the (loopI+II mut,AUG) TIR still used wild-type ribosomes fairly efficiently; its activity in the presence of ASD WT ribosomes was only threefold lower than that of the wild-type TIR. We therefore attempted to further manipulate the TIR so that its activity with wild-type ribosomes becomes more stringently dependent upon the presence of the GGA ele-

ments. To this end, the rpsA initiation codon was changed to GUG or UUG. The rationale here is that when the initiation codon is changed from an AUG to a non-AUG codon, the activity of the TIR generally becomes more dependent upon other elements, i.e., the SD or translational enhancers (34).

In the presence of ASD WT ribosomes, changing the initiation codon AUG to GUG, and, even more so, to UUG, affected the activity of the TIR very negatively (by as much as 45-fold for UUG) (Fig. 4B). Importantly, this activity was now more dependent upon the presence of the GGA motifs in loops I and II; when the initiation codon was GUG or UUG, the introduction of the (loopI+II) mutation reduced TIR activity about sixfold, instead of threefold when the initiation codon was AUG (Fig. 4B). Like for the wild-type rpsA TIR, the presence of ASD mut ribosomes had little effect on the activity of the (loopI+II WT,GUG) or (loopI+II WT,UUG) TIR. In contrast, when the loopI+II mutation was also present, ASD mut ribosomes stimulated the TIR activity, as for the (loopI+II mut,AUG) TIR. However, stimulation was now larger: the (loopI+II mut,GUG) and (loopI+II mut,UUG) TIRs were stimulated 2- and 4.5-fold, respectively, versus 1.3-fold for the (loopI+II mut,AUG) TIR (Fig. 4B).

Whereas these results show that rpsA TIRs carrying the loopI+II mutation are preferentially recognized by ASD mut ribosomes, they do not prove that this recognition reflects a pairing with the apical CCU motifs. Indeed, we noticed a possible slipped base pairing between the ASD mut and the AACC sequence present just before hairpin III, at nt +16 to −12 with respect to the initiator codon (Fig. 2B). To clarify the role of this sequence, we either deleted the CC doublet at nt −13 and −14 or replaced it by AU in the (loopI+II mut,UUG) TIR, for which the effect of specialized ribosomes was largest (ΔCC and CC→AU in Fig. 1 and 4B). As a control, the same mutations were also introduced in the (loopI+II mut,AUG) TIR. In all cases, in the absence of the ASD mut ribosomes, alterations of the CC doublet significantly increased β-galactosi-
da synthesis, indicating that the CC doublet acts as a negative element in the rpsA TIR. More importantly, these alterations also completely abolished the stimulation of the (loopI+II mut,UUG) TIR by ASD mut ribosomes (Fig. 4B). This result indicates that the observed stimulation reflects the presence of a spurious SD-like sequence that can pair with the ASD mut; in its absence, the loopI+II-mutated TIR is not stimu-
lated by ASD mut ribosomes.

This lack of stimulation may reflect a conformational difference between the loopI+II WT and loopI+II mut TIRs that renders the CCU trinucleotides unavailable for pairing. However, the mfold algorithm (26, 45) predicts that the secondary structure of the rpsA TIR does not vary upon changing the GGA triplets to CCU. To further document this point, the in vitro structures of the wild-type and (loopI+II mut,AUG) TIRs were probed with lead(II). This reagent preferentially recognizes and cleaves single-stranded regions, largely independently of their sequence (18, 24). Consistent with theoretical predictions, this technique revealed no difference in structure between the wild-type and loopI+II mut TIRs (Fig. 5). In particular, the CCU triplets appear to be fully accessible in the mutant.

Altogether, these experiments show that the conserved
GGA motifs in loops I and II do not act as a discontinuous SD element.

**DISCUSSION**

The role of two conserved GGA motifs in the rpsA TIR. As noted in the introduction, the rpsA TIR is unique in being highly structured (Fig. 1) and in requiring this conserved structure for high translational activity (3). One possibility is that the structure serves to present sequence elements optimally for interaction with the 30S subunit during initiation. In particular, two conserved GGA motifs, located in apical loops I and II of the rpsA TIR, are required for maximal activity of the TIR, and chemical probing suggests that they are largely accessible for a possible interactions with the 30S subunit (Fig. 5) (3). Taking advantage of established specialized ribosome systems in E. coli, we have addressed the nature of this interaction here. Because the two GGA triplets resemble SD elements, we have targeted the anti-SD sequence of the 16S rRNA as the putative interacting site. These triplets were changed from GGA to CCU, a change that, depending upon the construct used, depresses the activity of the TIR three- to sevenfold (Fig. 4B) without perturbing its structure, as judged from theoretical predictions (45) and structure-probing experiments (Fig. 5). However, the symmetrical change of the ASD from CCUCC UUA-3' (ASDWT) to GGAGGUUA-3' (ASDmut) did not bring any specific stimulation of the mutated TIR, except for an adventitious SD-like stimulation involving an AACCU motif located 12 to 16 nt upstream of the rpsA initiation codon (Fig. 2B and 4B). As a control, when a similar change [GGAG(C) to CCUCC] was introduced into the SD of the galE TIR, which depends stringently upon its SD for activity, the residual activity was stimulated by ASDmut ribosomes by as much as 600%. Altogether, the most straightforward interpretation of our data is that the two apical GGA motifs from loops I and II of the rpsA TIR do not form a discontinuous SD element, as initially proposed (3). Since the vestigial SD in stem III is also dispensable for activity (3), it seems plausible that the natural rpsA TIR does not rely at all upon an SD-ASD interaction for translation initiation.

Why, then, are the apical GGA motifs required for high translational activity? They may constitute interacting sites for regions of 16S rRNA other than the anti-SD or for a protein(s) from the translational machinery. Among the latter, one candidate is protein S1 itself. Ribosome-bound S1 is essential for the translation of most or all cellular mRNAs (39). Intriguingly, the RNA aptamer that binds S1 most tightly consists of a pseudoknot carrying a GGA motif in one of its loops (32). We speculate that, analogously, the GGA motifs from loops I and II may constitute recognition sites for ribosome-bound S1, allowing the correct positioning of the initiator codon to the P site. That purine-rich loops may constitute S1 binding sites has been suggested by others (40). Interestingly, the rpsA TIR is unique in that its activity is strongly repressed by free S1 (autoregulation) (2). Chemical protection analyses have identified s1, ss2, and part of loop III as targets for free S1, but the binding sites of free S1 and ribosome-bound S1 may not coincide. The susceptibility of the GG residues from both loops I and II to chemical modification increases in the presence of free S1, suggesting that the loops then undergo a conforma-

![FIG. 5.](http://jb.asm.org/) Compared patterns of lead(II)-induced cleavages in rpsA TIRs carrying either loop1+IIWT or loop1+IImut (Fig. 1). (A) In vitro-transcribed wild-type (WT) and (loop1+IImut,AUG) TIRs were probed with 60, 80, and 100 mM lead(II). Ctr, control without lead(II). L, alkaline hydrolysis ladder. Positions of the stem-and-loop regions of hairpins I and II and of single stranded regions (ss1 and ss2) are indicated. The smaller panels (right) focus on the apical loops of hairpins I and II; the relevant sequence is indicated on the right side, and uppercase is used for nucleotides from the loops. (B) The cleavage pattern of the region encompassing hairpins I and II is represented on the secondary structure model of the rpsA TIR (3). Strong, average, and weak cleavages are indicated by black, gray, and dotted arrows, respectively.
tional change (3). Possibly, this conformational change prevents recognition of the TIR by ribosome-bound S1.

Specialized ribosomes can translate bulk cellular mRNA. Under the conditions used here, the ASDmut 16S rRNA represents ca. 35% of total 16S rRNA, yet growth is not severely affected. This observation is consistent with that of Lee et al. (23), who found that cells expressing the same ASDmut 16S rRNA at a level similar to that used here were viable (clone 6 in reference 23). We also found that the ASDmut ribosomes are fairly efficiently enrolled in translation, even in the absence of a specific, complementary mRNA; their proportion in polysomes reaches 45% of their proportion in free 30S particles. Yet, the pattern of protein synthesized is similar to that observed in cells expressing the ASDWT ribosomes. Together, these two results suggest that ASDmut ribosomes participate in the translation of many cellular mRNAs and that, on the average, they are discriminated against by a factor of only about 2 in translation initiation. This observation was confirmed directly by using conditions where only ASDmut ribosomes (or ASDWT ribosomes) are active, i.e., in the presence of spectinomycin. Under these non-steady-state conditions, the patterns of protein synthesized by the ASDmut and ADSWT ribosomes were not identical, but quantitatively, protein synthesis by ASDmut ribosomes was still about one-half of that for ASDWT ribosomes.

How can ASDmut ribosomes translate bulk cellular mRNAs in the absence of a cognate SD-ASD interaction? A fraction of these mRNAs may spuriously carry SD-like elements with complementarity to ASDmut. The triplets ACC, UCC, and CCU all potentially bind the ASDmut with significant free energy (−1.2 to −1.8 kcal/mol [13]). Assuming that these triplets are not discriminated against in natural TIRs, about 50% of the TIRs will, on a random basis, carry one of them 4 to 15 nt upstream of the initiation codon, i.e., in a position where it may act as a minimal SD towards ASDmut ribosomes. As an illustration, the rpsA TIR itself carries an AACCU sequence that apparently can interact proficiently with ASDmut ribosomes (Fig. 4B). Alternatively, it is possible that cellular ribosomes, when present in the cell together with ASDmut ribosomes, can channel them into translation. Indeed, if an essential role of the SD-ASD interaction is to open inhibitory local secondary structures (8, 40), then after initiation by a ribosome with a wild-type ASD, opening may last long enough for ribosomes lacking such an ASD to initiate translation. Finally, it is possible that a significant fraction of all cellular mRNAs relies only marginally, if at all, on the SD-ASD interaction for translation, as exemplified here by the rpsA mRNA itself. Such mRNAs would make no distinction between wild-type and specialized ribosomes. There is, in fact, much evidence supporting the view that the SD-ASD interaction is not always required for efficient translation. In vitro, this interaction is clearly dispensable (6, 27). In vivo, our observation that translation of bulk cellular mRNAs remains significant when only ASDmut ribosomes are active in the cell is not unprecedented (15, 17; see Fig. 5, lanes 6 to 8, in reference 17), although this point was not emphasized in these previous studies. Other arguments support the idea that SD-independent translation is not marginal in E. coli. Although highly expressed genes generally possess stronger SDs than average ones, as many as 12% of E. coli mRNAs lack a recognizable SD (25). Consistent with the dispensability of the SD interaction, artificial mRNAs lacking an SD in their leader sequences (42), and even leaderless mRNAs (22), can be translated in E. coli. Even for TIRs with a bona fide SD, cases are known where alterations in the SD do not abrogate or even do not affect activity, in spite of the loss of the SD-ASD interaction (12, 19, 23). Thus, the situations exemplified here with the galE and rpsA TIRs (stringent dependence or no dependence upon the SD-ASD interaction, respectively) may simply constitute extreme cases of a more common situation where the SD-ASD interaction is only one element among others contributing to translation initiation.

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