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Protein S1 counteracts the inhibitory effect of the extended Shine–Dalgarno sequence on translation

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ABSTRACT

There are two major components of *Escherichia coli* ribosomes directly involved in selection and binding of mRNA during initiation of protein synthesis—the highly conserved 3′ end of 16S rRNA (aSD) complementary to the Shine–Dalgarno (SD) domain of mRNA, and the ribosomal protein S1. A contribution of the SD-aSD and S1-mRNA interactions to translation yield in vivo has been evaluated in a genetic system developed to compare efficiencies of various ribosome-binding sites (RBS) in driving *β*-galactosidase synthesis from the single-copy (chromosomal) *lacZ* gene. The in vivo experiments have been supplemented by in vitro toeprinting and gel-mobility shift assays. A shortening of a potential SD-aSD duplex from 10 to 8 and to 6 bp increased the *β*-galactosidase yield (four- and sixfold, respectively) suggesting that an extended SD-aSD duplex adversely affects translation, most likely due to its redundant stability causing ribosome stalling at the initiation step. Translation yields were significantly increased upon insertion of the A/U-rich S1 binding targets upstream of the SD region, but the longest SD remained relatively less efficient. In contrast to complete 30S ribosomes, the S1-depleted 30S particles have been able to form an extended SD-aSD duplex, but not the true ternary initiation complex. Taken together, the in vivo and in vitro data allow us to conclude that S1 plays two roles in translation initiation: It forms an essential part of the mRNA-binding track even when mRNA bears a long SD sequence, and through the binding to the 5′ untranslated region, it can ensure a substantial enhancing effect on translation.

Keywords: BoxA; ribosomal protein S1; RNA–protein interactions; Shine–Dalgarno interaction; translation initiation

INTRODUCTION

The problem of correct recognition of the translation start by the small ribosomal subunit is differently solved in eukaryotes and prokaryotes (reviewed by Jackson, 2000). In the case of prokaryotic translation initiation, ribosomes are able to distinguish the initiator AUG (or non-AUG) codon from synonymous triplets throughout the mRNA due to the presence of specific signals in the vicinity of the translation start (Schneider et al., 1986; Gold, 1988; Dreyfus, 1988; Gualerzi & Pon, 1990; Ringquist et al., 1992). For the vast majority of *Escherichia coli* and bacteriophage mRNAs, the major characteristic of the ribosome binding site (RBS) is the Shine–Dalgarno (SD) domain, which base pairs with the complementary sequence (aSD) near the 3′ end of 16S rRNA during initiation complex formation (Shine & Dalgarno, 1974; Steitz & Jakes, 1975; Hui & de Boer, 1987; Jacob et al., 1987). At the same time, the SD interaction was found to be not essential for correct initiation (Calogero et al., 1988; Melançon et al., 1990), and an existence of functional mRNAs completely lacking SD indicates that prokaryotic ribosomes have other capacities for start site selection. Typical examples of this kind include leaderless messengers (Shean & Gottesman, 1992; Wu & Janssen, 1997; van Etten & Janssen, 1998), mRNAs bearing the plant viral leaders (Wilson, 1986; Gallie & Kado, 1989; Tzareva et al., 1994), and the *tuf* mRNA of *Mycoplasma genitalium* (Loechel et al., 1991), which are correctly recognized

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and translated by *E. coli* ribosomes despite the absence of any SD-like sequence upstream of the start codon. Moreover, the SD-independent initiation pathway appeared to be preferentially used by the prokaryotic-like translational machinery of chloroplasts, although the aSD sequence at the 3′ end of chloroplast 16S rRNAs is highly conserved (Fargo et al., 1998, 1999). The long 5′-untranslated leaders of the chloroplast mRNAs are able to drive efficient translation in *E. coli*, thus supporting the existence of intrinsic SD-independent mechanism(s) for ribosome recruitment in this bacterium and its relatives. Molecular interactions underlying such noncanonical translation initiation in *E. coli* are most likely involved also in the classic SD-mediated initiation process, but in the presence of typical initiation signals, their role is masked and hence underestimated.

Besides the SD domain, several mRNA *cis* elements were found to have a substantial positive effect on the translation efficiency, presumably due to its direct involvement in ribosome recruitment at the initiation step (McCarthy & Gualerzi, 1990; McCarthy & Brimacombe, 1994). Among positive *cis* elements, first of all, one should note the so-called translational enhancers located within mRNA untranslated leaders (McCarthy et al., 1985; Gallie & Kado, 1989; Olins & Rangwalla, 1989; Zhang & Deutscher, 1992), and the downstream box (DB) found at the beginning of coding sequences in many *E. coli* genes (Shean & Gottesman, 1992; Sprengart et al., 1996; Etchegaray & Inouye 1999). Both of these *cis* elements were regarded as responsible for SD-independent pathways of initiation complex formation on SD-less mRNAs (e.g., Gallie & Kado, 1989; Loechel et al., 1991; Shean & Gottesman, 1992; Golshani et al., 2000), and in both cases complementary interactions with certain regions within 16S rRNA were postulated to account for the enhancing effect (Olins & Rangwalla, 1989; Sprengart et al., 1996). However, recent studies present convincing arguments against the proposed mRNA–rRNA base pairing models (O'Connor et al., 1999; O'Connor & Dahlberg, 2001; Moll et al., 2001), suggesting that enhancing effects are based on other molecular mechanisms.

According to an alternative model, translational enhancers within the mRNA 5′ untranslated regions (5′ UTR) serve as targets for a key mRNA-binding ribosomal protein S1 (Boni et al., 1991; Zhang & Deutscher, 1992). This unusual ribosomal protein (Subramanian, 1983), essential for the translation machinery of Gram-negative organisms but not for ribosomes from most Gram-positive species, ensures the known ability of *E. coli* to translate mRNAs from a wide variety of sources, regardless of the SD sequence length (Roberts & Rabinovitz, 1989; Farwell et al., 1992; Tzareva et al., 1994). Moreover, this protein is indispensable for translation of any *E. coli* messenger in vivo (Sørensen et al., 1998). It was shown that S1 interacts with single-stranded regions within mRNA leaders (5′ to the SD when it is present) during translation initiation complex formation in vitro (Boni et al., 1991; Tzareva et al., 1994). Very recently, this was directly confirmed by cryoelectron microscopic studies (Sengupta et al., 2001). SELEX experiments have shown that S1 is the major component of the *E. coli* ribosome directly involved in mRNA selection, and that the RNA-binding specificity of free S1 is quite the same as that of S1 within the 30S subunit (Ringquist et al., 1995). One more important and rather unexpected result has been obtained by SELEX: It turned out that intact (S1-containing) 30S ribosomes do not select RNA aptamers with extended SD sequences, whereas S1-depleted 30S particles select only this kind of ligands. This puzzling fact implies that the primary binding of an mRNA by native, S1-containing 30S ribosomes in vivo may also be driven by S1 rather than by the SD-aSD interaction. At the same time, SELEX is the in vitro technique that generates high-affinity RNA ligands irrespective of their in vivo activity, and so the correlation between high-affinity 30S-mRNA binding and translation yield is by no means evident. The goal of the present study is to evaluate the relative contributions of RNA–RNA (SD-aSD) and RNA–protein (mRNA-S1) interactions to translation in vivo.

Earlier, the inhibitory effect (~40%) of long SD sequences on translation yield from the plasmid-encoded mRNAs was observed by de Boer et al. (1983), but since then, this problem has not been thoroughly studied, and the premise that initiation efficiency is related to the number of complementary base pairs between the SD and aSD regions is widely held (see Jackson, 2000). To elucidate the role of the strength of SD interactions in vivo, we have compared activities of RBSs differing only in the length of the SD motif in driving translation from the chromosomal (single-copy) *lacZ* gene, and found that an extended (10 nt) SD sequence is much less efficient than the shorter ones. The translation yield can be substantially increased after insertion of a good S1-binding site upstream of SD. As a target for S1, we took the *E. coli rrnB BoxA* sequence. Recently, Mogridge and Greenblatt (1998) reported that this transcriptional antiterminator *cis* element binds S1 in vitro specifically and strongly, and so it was of interest to test whether this reliable S1 target can serve as a translational enhancer when placed within mRNA leaders. Our results confirm that S1-binding regions upstream of SD sequences affect translation very positively. We conclude that S1 targets within mRNA leaders are much more favorable for efficient translation than extended SD sequences, because a too stable SD-aSD duplex can negatively affect the kinetics of the translation process. The results obtained are fully consistent with the fact that *E. coli* mRNAs encoding abundant cellular proteins (e.g., ribosomal proteins), do not, as a rule, contain strong SD domains, but bear fairly long leaders comprising potential S1-binding sites.

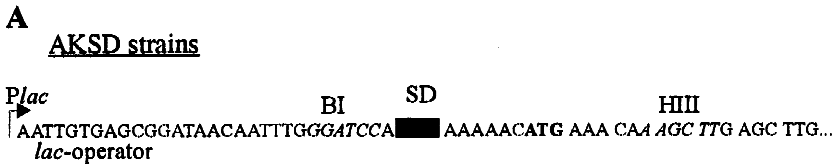
RESULTS

The longest SD sequence is the least efficient in translation in vivo

As shown by SELEX, the native S1-containing 30S ribosomes, in contrast to the S1-depleted 30S, do not select RNA ligands bearing extended SD motifs, although they can bind them in vitro with a high affinity (Ringquist et al., 1995). To find out whether an extended SD sequence 5′-AAGGAGGUGA from the high-affinity ligands generated against the S1-depleted 30S is able to drive efficient translation in vivo, we have exploited the genetic system, allowing us to compare the strengths of various RBSs (artificial or natural) by measuring their capacities to drive translation from the fully induced single-copy (chromosomal) *lacZ* gene (Dreyfus, 1988; Yarchuk et al., 1992; Boni et al., 2000, 2001). Thus, the results obtained are independent of transcription effects and of gene dosage.

To design artificial RBSs differing only in the length of the SD sequences (Fig. 1), we took into account the data on the optimal sequence/structure elements within prokaryotic initiation regions (Dreyfus, 1988; Gold, 1988; Ringquist et al., 1992). Accordingly, our RBSs possess an A-rich context, a classic AUG start codon, and a six-base spacer separating an SD domain from the start (Fig. 1). Synthetic DNA duplexes corresponding to the RBSs comprising 10-, 8- and 6-nt-long SD motifs were cloned in pEMBLΔ46 vector (Dreyfus, 1988) in phase with the *lacZ* coding sequence and then transferred onto the *E. coli* chromosome by homologous recombination. In the resulting strains, β-galactosidase synthesis was driven at the transcription level by the *lac* promoter-operator region ensuring the same promoter strength, and at the translation level—by our artificial RBSs (Fig. 1A). According to Zuker's algorithm (http://bioinfo.math.rpi.edu/~mfold), the 5′ regions of the corresponding mRNAs do not contain significant secondary structures inhibitory for ribosome binding during translation initiation; in particular, SD regions and the start codon are not involved in stable base pairings (Fig. 1B).

The β-galactosidase assay revealed that the RBS with the longest complementarity to the 3′ end of 16S RNA directed the least efficient protein synthesis (Fig. 1A). The highest translation yield was obtained for the 6-nt AAGGAG sequence that turned out to be even more efficient than the 8-nt AAGGAGGU SD domain. Because theoretical predictions argue against considerable alterations in intramolecular structure of 5′ mRNA regions upon reduction of the SD length, we suppose the observed differences in translation yield to be determined by the SD-aSD duplex stability. Indeed, the ΔG values for SD-aSD duplex formation are −15.2, −11.1, and −6.1 kcal/mol for 10-, 8-, and 6-nt SD sequences, respectively (calculated according to Frier et al., 1986). Taking into account that at a certain mo-



strain	SD	β-galactosidase activity
AKSD 10	AAGGAGGUGA	1250± 200
AKSD 8	AAGGAGGU	5200± 300
AKSD 6	AAGGAG	7200± 400

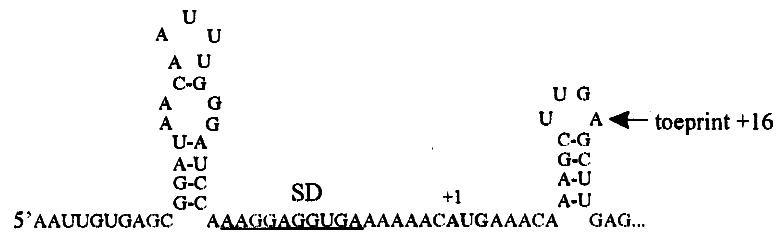


FIGURE 1. Schematic representation of the 5′ regions of chromosomal RBS-*lacZ* fusions bearing the SD elements of different length (**A**), and the predicted secondary structure of the 5′ region of the mRNA bearing the longest SD sequence (**B**). β-galactosidase activities in AKSD strains (**A**) are expressed in nanomoles of ONPG hydrolyzed per minute per milligram of total soluble cell proteins. The mRNA start is indicated by an arrow; the ATG start codon is in bold; BI and HIII are *Bam*HI and *Hin*dIII sites (italicized); the SD sequence is marked by a filled box. Measurements of β-galactosidase activity in cell lysates were performed for at least four independent cellular cultures (see Materials and Methods).

ment during elongation, the SD duplex should be melted to clear the RBS for the subsequent initiation event, we suggest that too strong SD interactions may negatively affect translation by increasing the clearing time (taken for the ribosome to clear the RBS for the next initiating 30S subunit).

Ternary initiation complex formation for artificial RBSs bearing the least (10-nt) and the most (6-nt) efficient SD sequences (see Fig. 1) was checked in vitro by toeprinting according to Hartz et al. (1988). To reproduce the complete 5′ region of the mRNA synthesized in vivo, we included the *lac* operator in the constructs for RNA synthesis in vitro (see Materials and Methods). Both mRNAs in the presence of the native (S1-containing) 30S subunits and initiator tRNA generate comparable toeprint signals (Fig. 2), suggesting that a difference of more than sixfold in translation yield in vivo (Fig. 1A) cannot be ascribed to their different capabilities to form the ternary initiation complex. It should be noted that S1-depleted 30S particles cannot form a true ternary complex able to stop primer extension at the classic toeprint position +16 (with respect to the A + 1 of the start codon; see Fig. 1B), but they are certainly able to bind an mRNA by the SD interaction, as, in the case of the 10-nt SD sequence, they generate a distinctive double signal at positions −8, −9 corresponding to the 3′ edge of the SD-aSD duplex (Fig. 2A). Although AMV reverse transcriptase used in toeprinting generally can pass through RNA structures, in the case of their high stability, an arrest of cDNA synthesis is often observed (see Hartz et al., 1991b). Thus, in the case of S1-depleted particles forming the heteroduplex with the 10-nt SD sequence, the enzyme hardly penetrates further than positions −8, −9. Remarkably, these signals (corresponding to the 3′ boundary of the SD-aSD duplex formed in the absence of tRNA) are not generated by native, S1-containing 30S particles (Fig. 2A). It means that S1 within 30S somehow prevents formation of the full-size mRNA-16S RNA duplex able to arrest the AMV reverse transcriptase. This suggestion is supported by the fact that, in the case of the 6-nt SD sequence (Fig. 2B, the lane with S1-depleted 30S), a stop signal at the 3′ edge of the SD duplex is not observed, implying that 6 bp in a heteroduplex are hardly sufficient to arrest reverse transcription in our conditions. The results of toeprinting are consistent with the SELEX data demonstrating that extended SD sequences are selected only by 30S lacking S1 (Ringquist et al., 1995).

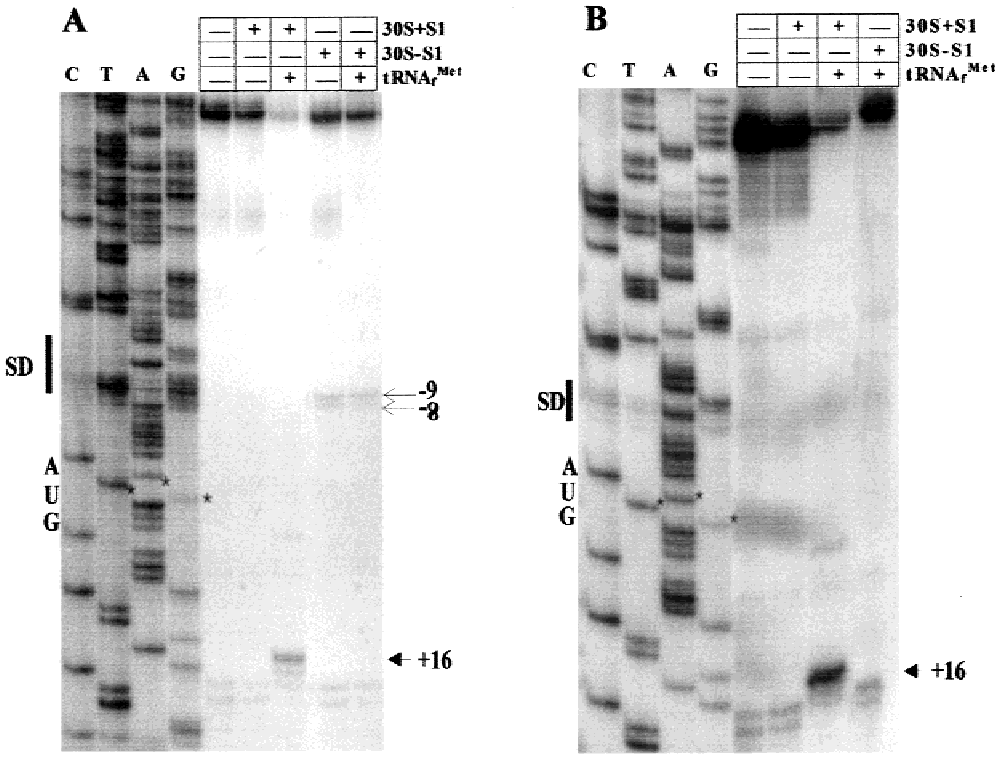


FIGURE 2. Extension inhibition analysis (toeprinting) of the binary and ternary complex formation on the mRNAs comprising 10-base (**A**) and 6-base (**B**) SD regions. A, G, C, and T: sequence lanes resulting from the plasmid sequencing (**A:** pSD10, **B:** pSD6) with the same primer DSlac that was used for toeprinting. The absence (−) or presence (+) of the 30S ribosomes (S1-containing or S1-depleted) and initiator tRNA in reaction probes are shown above the lanes. The signal from the 3′ edge of the SD duplex in a binary complex with S1-depleted 30S particles is marked by thin arrows, the classic toeprint signals generated by native 30S subunits in the presence of initiator tRNA by thick arrows, the AUG start codon on the sequence lanes by asterisks, and the SD sequence by vertical bars.

We conclude that the presence of S1 can prevent the formation of the too stable duplex able to trap completely the ribosome on the RBS. Such a trap is predicted by theoretical calculations that show that an RNA duplex of 10 bp has a half-time for dissociation of ~30 min (at 30 °C), and G/C-rich duplexes of 10 bp have dissociation half-times up to 100 years (see Herschlag, 1995). Obviously, it would impede the transition from initiation to elongation and hence the measurable production of β-galactosidase. If, nevertheless, the synthesis is relatively low but productive (Fig. 1), this implies that the full-length 10-bp SD duplex is not formed in the presence of S1.

BoxA, a transcriptional antiterminator of the *E. coli rrn* operons, acts as a translational enhancer when placed within 5′ UTR upstream of the SD region

Although the above results suggest that S1 is able to decrease the number of base pairs between the 10-nt SD region and the 16S RNA 3′ end, that is, to modulate SD interactions, even in this case, the longest SD appears much less efficient in vivo than the shorter ones (Fig. 1A). In an attempt to improve the efficiency of translation by facilitating the S1 binding with the 5′ region, we inserted a sequence representing an efficient S1-binding site upstream of the SD domain in each construct. Recent experiments of Mogridge and Greenblatt (1998) showed that *BoxA*, a transcriptional antiterminator *cis* element of the *E. coli rrn* transcripts, binds S1 in vitro strongly and specifically. It was therefore interesting to test this reliable S1-binding site for its ability to enhance translation. To provide controls for the specificity of the S1-*BoxA* interaction, we made similar constructs with the sequence complementary to *BoxA* (*compBoxA*) and with the mutated *BoxA* (*mutBoxA*).

The β-galactosidase assay revealed that both *BoxA* and *compBoxA* significantly enhance translation, with the highest relative effect being observed for the longest SD (Fig. 3). Interestingly, this enhancement cannot be detected at the level of initiation complex formation in vitro, as no visible quantitative or qualitative difference is observed using the toeprinting assay (Fig. 4A). It suggests that this technique does not always reflect the in vivo translation capacity of an mRNA. At the same time, for other mRNAs, the efficiency of initiation complex formation in vitro is dependent on S1 targets

strain	SD	β-galactosidase activity
BASD 10		8400 ± 450
CompBASD 10	AAGGAGGUGA	9700 ± 1100
MutBASD 10		2700 ± 300
BASD 8	AAGGAGGU	11350 ± 600
CompBASD 8		11300 ± 600
BASD 6	AAGGAG	11000 ± 1200
CompBASD 6		12600 ± 1400

FIGURE 3. The 5′-terminal *lac*-regions in BASD, CompBASD, and MutBASD strains and corresponding β-galactosidase activities measured in clarified cell lysates (in nanomoles of ONPG hydrolyzed per minute per milligram of total soluble cell proteins). The *rrnB BoxA*, its complementary sequence (*compBoxA*), and the *mutBoxA* sequence are open boxed, the changes introduced in the *BoxA* sequence and its right flank (MutBASD strain) are encircled, and other designations as in Figure 1A.

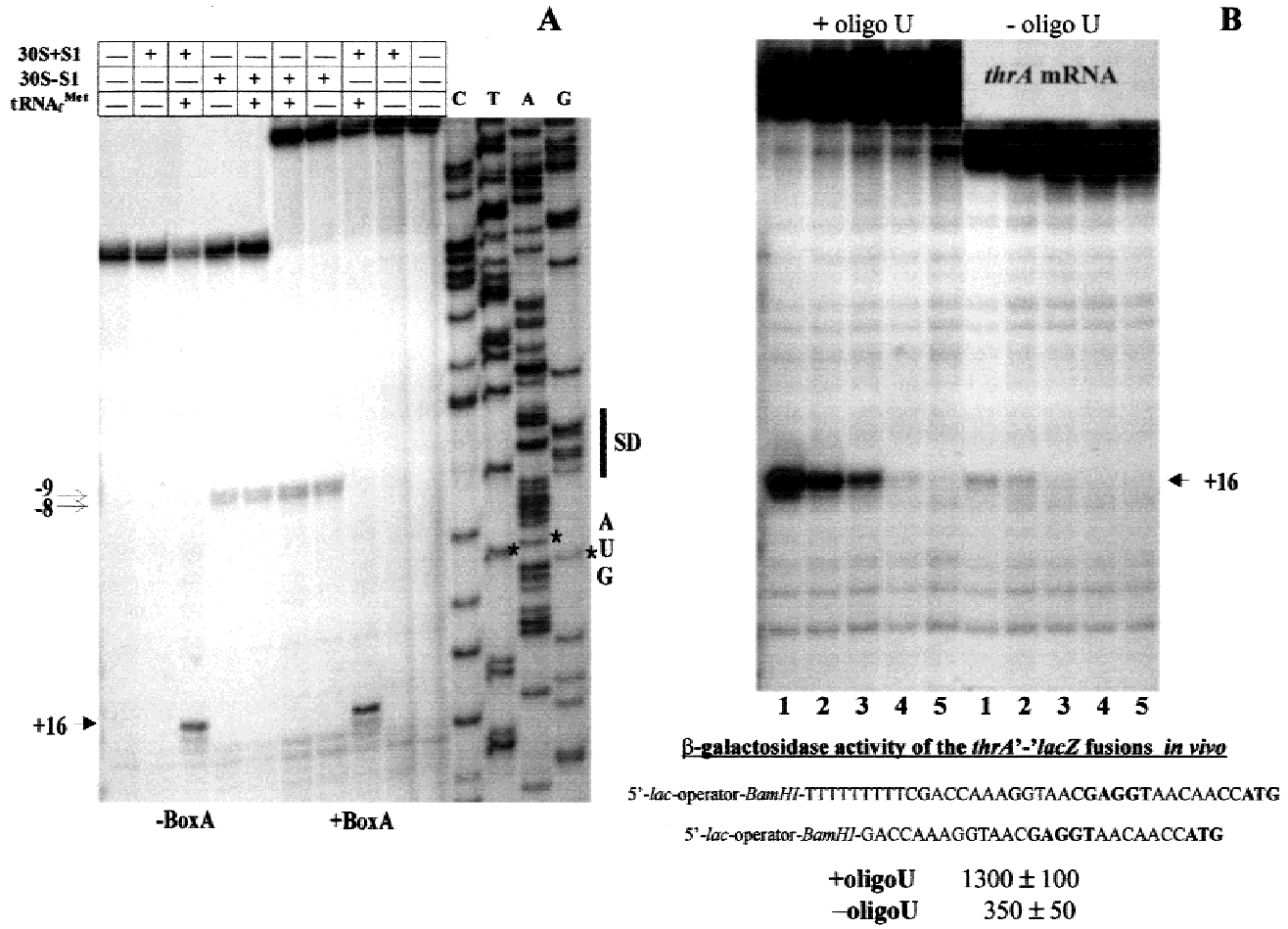


FIGURE 4. Dependence of the efficiency of initiation complex formation in vitro upon the presence of S1 targets within the mRNA leader. **A:** Efficiency of the initiation complex formation on the mRNA bearing the 10-base SD sequence is not dependent on the presence of *BoxA* upstream of SD. Toeprint analysis of the binary and ternary initiation complexes formed on the mRNAs comprising (+) or not comprising (−) *BoxA* within the leader. See the legend to Figure 2. **B:** Efficiency of the initiation complex formation on the *thrA* mRNA in vitro is dependent on the presence of an oligoU stretch within the leader. Toeprint analysis of ternary initiation complex formation at decreased concentration (micromolar) of 30S ribosomes (lanes 1–5: 0.8, 0.4, 0.2, 0.1, and 0.05 μM 30S, correspondingly).Concentration of initiator tRNA in all probes is 4 μM. Below the gel: The structure of 5′ regions of the *thrA'–'lacZ* fusions and β-galactosidase activities in corresponding ENSO strains.

to a higher extent. Thus, the deletion of the S1 target represented by an oligoU stretch from the leader of the *thrA'–'lacZ* fusion (Boni et al., 2000) leads not only to an approximately fourfold drop in the β-galactosidase production in vivo but also to a significant decrease in the *thrA* RBS capacity to form the ternary initiation complex in vitro (Fig. 4B). Obviously, the S1-oligoU binding contributes much to the *thrA* RBS-30S interaction within the initiation complex. We suppose that in the case of the *BoxA*-containing mRNA, an S1 target upstream of SD may serve in vivo as a transit site for ribosome landing.

The results obtained (Fig. 3) indicate that the *BoxA*-mediated translational enhancement is not related to the known ability of *BoxA* to increase transcription rate or to ensure antitermination of transcription (see Vogel & Jensen, 1995), as the *compBoxA* affects the translation yield in a similar way. Moreover, the fact that both inserts can serve as translational enhancers allows us to exclude their putative complementary interactions with 16S RNA. We believe that the enhancing effect is most likely explained by the S1-mediated preferential binding of the 30S ribosome to the A/U-rich sequences within the mRNA leaders. Indeed, both *BoxA* and *compBoxA* are equally rich in A/U residues suggesting that *compBoxA* also represents a potential S1 target, that is fully consistent with the known fact that S1 has no strict sequence preferences (Subramanian, 1983). It should be mentioned that the absence of strict sequence preferences does not mean the absence of specificity at all. When we introduced point mutations in the *BoxA* insert by altering several C, U, and A residues for G (Fig. 3, MutBASD strain), this gave only a modest increase in translation yield, in comparison with the effect of *BoxA* or *compBoxA*. Three of these alterations (within the *BoxA* sequence) were previously shown to de-

crease the affinity of S1 for *BoxA* (Mogridge & Greenblatt, 1998). These data correlate well with the reduced affinity of S1 for G-rich sequences (Subramanian, 1983; Ringquist et al., 1995).

Our assumption that the insertion of *BoxA* or *compBoxA* within the mRNA leaders enhances translation by favoring the S1-mediated 30S binding was examined in vitro using the gel-mobility shift assay (Fig. 5). Because the RNA-binding specificity of free S1 is the same as that of S1 within the 30S subunit (Ringquist et al., 1995), the affinity of S1 to the mRNA leader should correlate with the in vivo translation activity of the messenger. In accordance with this statement, the band shifting shows that S1 binds *BoxA*- or *compBoxA*-comprising leaders more tightly than the leader bearing the mutated *BoxA* or lacking any insert (Fig. 5). Indeed, at the S1 concentration sufficient to bind completely the *BoxA*- or *compBoxA*-RNAs (Fig. 5A–C, lane 2), the *mutBoxA*-RNA is bound only partially. It should be mentioned that all three RNA species have the same length (86 nt) and are present at the same low concentration (4–5 nM). The lower affinity for S1 (in comparison with Fig. 5A,B) was also observed for the 5′ region of the *lacZ* mRNA from AKSD10 lacking any insert (Fig. 5D). To show that the lower affinity is not caused by the shorter length of the leader, the RNA used in the band-shift assay was extended downstream from the start codon up to 130 nt in length (see Materials and Methods). One can notice that even at the highest S1 concentration, this RNA is bound only partially. The results of the mobility-shift assay show that the mRNA translational activity in vivo directly correlates with the affinity of its 5′ region for S1, and even small differences in affinity may significantly affect the efficiency of translation in vivo.

The enhancing effect of *BoxA* or *compBoxA* on translation is not surprising. Indeed, both elements are extended A/U-rich sequences and, in this respect, they are reminiscent of a number of known translational enhancers including the epsilon of the T7 gene 10 mRNA (Table 1). As was shown recently by O'Connor and Dahlberg (2001), an insertion of the epsilon upstream of the 8-nt SD sequence (AAGGAGGU) gives an approximately twofold increase in translation yield, that is, quantitatively about the same enhancing effect that we have obtained by inserting *BoxA* or *compBoxA* into the construct with the same 8-nt SD sequence (Figs. 1 and 3). Taking into account that the optimal site for binding of one S1 molecule was estimated to comprise 10–12 nt (Subramanian, 1983), we suggest that any single-stranded A/U-rich sequence of the appropriate length located upstream of SD can serve as a translational enhancer by favoring 30S binding through interaction with S1.

DISCUSSION

As shown previously, S1 ensures translation in *E. coli* of those mRNAs that lack the SD or bear a weak SD signal (Roberts & Rabinowitz, 1989; Farwell et al., 1992; Tzareva et al., 1994). The results of the present article demonstrate that S1 is also essential for binding and translation of mRNAs bearing long SD sequences. It is consistent with the observation of Sørensen et al. (1998) that in vivo translation of all *E. coli* mRNAs is dependent on S1. As we show here, a visible signal corresponding to the 3′ boundary of the strong SD duplex in a binary 30S-mRNA complex can be obtained only for the particles lacking S1, but not for the native S1-containing 30S, indicating that S1 is able to modulate

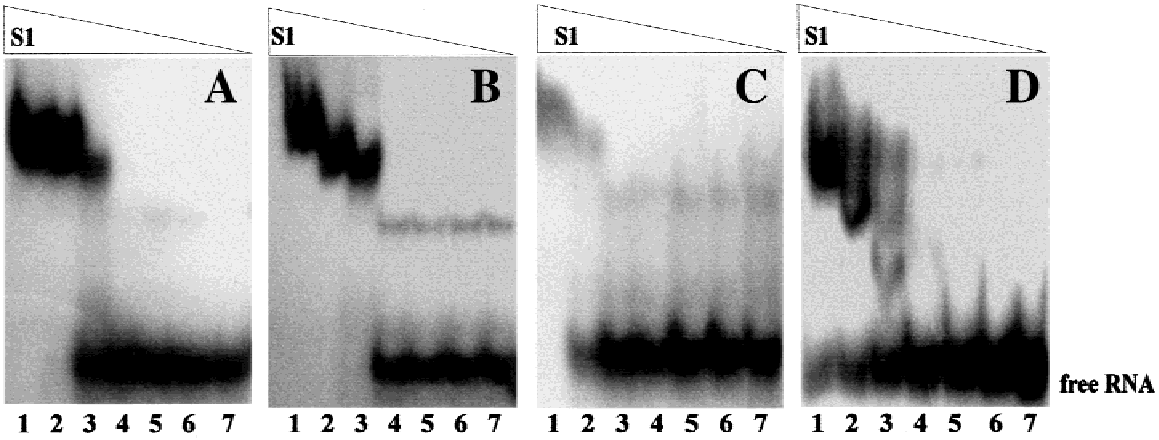


FIGURE 5. Affinity of S1 to the *BoxA*- or *compBoxA*-comprising mRNA leaders is higher than to the leader lacking these elements or bearing a mutated *BoxA* (*mutBoxA*, see Fig. 3 for sequences). [^{32}P-αUTP]-labeled RNAs produced by transcription in vitro correspond to the 5′ regions of the *lacZ* mRNA from the BASD10 (**A**), CompBASD10 (**B**), MutBASD10 (**C**), and AKSD10 (**D**) strains, and comprise 86 (**A**, **B**, **C**) and 130 (**D**) nucleotides. Gel-mobility shift assay (8% nondenaturing gel) was carried out at gradually decreased S1 concentrations (micromolar): 1.0 (lane 1), 0.5 (lane 2), 0.25 (lane 3), 0.125 (lane 4), 0.06 (lane 5), and 0.03 (lane 6); the last probe (lane 7) contains no S1. Concentration of RNA in all probes is 4–5 nM.

TABLE 1. Translational enhancers within the leaders of mRNAs.

Gene	Sequence (5′ → 3′)	Reference
atpE E. coli	UUUUAACUGAAACAAA	McCarthy et al., 1985
T7 gene10	UUUAACUUUAA	Olins and Rangwalla, 1989
T4 gene 32	UUAAAUUAAAA	Hartz et al., 1991a
rpsA E. coli	UUAAAUAUAAA	Boni et al., 2001
tuf M. genitalium	UUAACAACAUAAUUU	Loechel et al., 1989
Ω of TMV RNA	ACAAUUAC-repeats	Gallie and Kado, 1989
rrn BoxA E. coli	CACU**GCUCUUUAACAA**UUUAUCA[a]	This paper
CompBoxA	UGAUAAAUUGUUAAAGAGCAGUG	This paper

[a]The *rrnB BoxA* sequence (in bold) is supplemented with its natural 4-nt left and 7-nt right flanks. It should be noted that the left flank and the first 4 nt of the right flank were present in the construct of Mogridge and Greenblatt (1998) in their experiments on S1-*BoxA* binding in vitro.

the strength of the SD-aSD interaction (Figs. 2A and 4A). This ability of S1 most likely enables translation of mRNAs bearing the SD sequence as long as 10 nt despite the theoretical prediction of the extreme longevity for the RNA duplex of 10 bp that should completely trap the ribosome on the RBS (see Herschlag, 1995). In fact, such a duplex cannot be formed in the presence of S1. Moreover, the formation of the stable SD-aSD duplex (in the absence of S1) is not a guarantee of its conversion into a true ternary initiation complex upon addition of initiator tRNA (Figs. 2A and 4A); although we cannot completely exclude the possibility of the S1-independent ternary complex formation in some specific cases (e.g., see Balakin et al., 1992). This is consistent with the notion of Calogero et al. (1988) that the SD interaction is mechanistically irrelevant for 30S initiation complex formation.

Our results show that among the strong SD sequences comprising 6, 8, and 10 bases complementary to the 3′ terminus of 16S RNA, the shortest one appears to be the most efficient, giving the highest translation yield in vivo (Fig. 1). We suggest that the strong SD duplex may cause ribosome stalling at the initiation site, thus increasing the clearing time (the time taken for the ribosome to clear the RBS for the next initiation event) and slowing translation. The analogous situation has been described for transcription, where too tight contacts of *E. coli* RNA–polymerase with the consensus promoter elements cause the polymerase to stall as it begins to transcribe, thus slowing transcription (Ellinger et al., 1994). Although, as we have shown here, the translation yield can be substantially augmented upon insertion of the S1 binding targets upstream of SD (see below), the longest SD remains relatively less efficient.

Because an insertion of the S1 target in the mRNA leader is able to increase substantially the translation yield (Figs. 1 and 3), interaction of S1 with mRNA can be regarded as the first event in translation initiation in vivo, even when an mRNA bears a strong SD sequence. This enhancing effect is most likely determined by the fact that in prokaryotes, translation of the mRNA begins long before its transcription is over, and in many cases, transcription and translation are tightly coupled (for the *lacZ* case, see Yarchuk et al., 1992). In this situation, those mRNAs that are able to bind to the ribosome early after the beginning of transcription should have an advantage over others in competing for the ribosome. An elongated shape of the S1 RNA-binding domain connected to its ribosome-binding domain by a flexible hinge (Subramanian, 1983) gives S1 the ability to inspect a much larger area around the ribosome while searching for an mRNA than that available to the 3′ terminus of 16S RNA. Due to the location of S1 near the 3′ end of 16S RNA (Sengupta et al., 2001) and the flexibility between its two domains, the S1-mRNA primary contacts increase the local concentration of the SD region in the vicinity of the aSD sequence, thus promoting the SD interaction that, in turn, ensures a higher concentration of the start codon near the P-site. This scheme of interdependent events envisages that in some cases (functional mRNAs bearing leaders lacking SD; see Introduction) the stage of the SD interaction can be omitted, and a direct fitting of the start codon into the P-site can be provided by S1-mRNA contacts alone.

Although in this work we have used artificial constructs, the data obtained are fully consistent with the natural situation, as *E. coli* mRNAs encoding abundant cellular proteins never comprise very long SD elements, that is, they do not use extended mRNA–16SRNA interactions to attain high translation efficiency. Thus, for ribosomal protein mRNAs, an average length of the SD motif with contiguous complementarity to the aSD sequence is 4.4 nt (our estimation). Although these and other highly expressing mRNAs show no visible correlation between their translation efficiency and the number of complementary base pairs in the potential SD-aSD duplex, they share some important characteristics; in particular, they bear fairly long 5′ UTRs to ensure efficient and, in many cases (e.g., r-protein operons), regulated translation. Very often, 5′ truncation of natural mRNA leaders causes a drop in their efficiency, even if conventional RBSs (covering positions

from −20 to +15 relative to the start point; see Gold, 1988) remain intact (e.g., *atpE* mRNA, McCarthy et al., 1985; *galE*, Dreyfus, 1988; Boni et al., 2000; T7 genes 1 and 0.3, Fatscher et al., 1988; T4 gene 32, Hartz et al., 1991a; *thrS*, Sacerdot et al., 1998; *rpsA*, *thrA*, and *rplL*, Boni et al., 2000). We believe that most if not all 5′ UTRs of efficient mRNAs bear S1 targets providing efficient and rapid ribosome recruitment.

As to the putative functional role of S1 in recognition of *BoxA* within the *rrn* operon transcripts (Mogridge & Greenblatt, 1998), we still have rather few facts to discuss this problem in terms of models. Anyhow, it should be taken into account that not only free S1, but also S1 within the 30S subunit can take part in this recognition; therefore, the native 30S must be equally regarded as a potential participant of the *BoxA*-mediated process, whichever it is—regulation of antitermination, rRNA processing or any other.

MATERIALS AND METHODS

Strains, plasmids, and genetic methods

Strain XL1-blue (Stratagen) was used for plasmid propagation. All genetic constructions for measuring the β-galactosidase activity from the single-copy (chromosomal) *lacZ* gene were derivatives of the ENSO strain (formerly HfrG6Δ12; Dreyfus, 1988). The Lac⁻ phenotype of ENSO is conditioned by a deletion covering the promoter and RBS of *lacZ* (from −51 to +21, with respect to the translation start). The plasmid pEMBLΔ46 is a pEMBL8+ derivative in which a smaller *lac* region (from −15 to +21) has been replaced for multiple cloning sites. DNA fragments corresponding to the RBS of a choice are inserted in phase with the *lacZ* coding sequence of pEMBLΔ46 and then transferred onto the chromosome of ENSO by homologous recombination, selecting for the Lac⁺ phenotype (Dreyfus, 1988; Boni et al., 2000).

Construction of new plasmids and ENSO derivatives for *lacZ* expression

To insert artificial RBSs comprising 10-, 8-, and 6-base SD elements in front of the *lacZ* gene, three pairs of synthetic oligodeoxyribonucleotides, sd3-sd4, sd5-sd6, and sd7-sd8 (Table 2), were annealed to obtain DNA duplexes with overhanging 5′ ends for direct cloning into *Bam*HI and *Hin*dIII sites of pEMBLΔ46. Insertion of the RBSs reanimated the RBS-depleted *lacZ* gene of pEMBLΔ46, and so the transformants could be selected by α-complementation. The resulting pEMBL derivatives pSD10, pSD8, and pSD6 (where the numbers correspond to the length of the SD motif) were used to transfer the RBS-*lacZ* fusions onto the ENSO chromosome. Corresponding ENSO derivatives were named as AKSD (10, 8, and 6).

To insert the *BoxA* of the *E. coli rrnB* operon upstream of the SD regions of pSD, oligonucleotides BA and compBA (Table 2) were annealed to obtain a duplex with overhanging *Bam*HI (left) and *Bgl*II (right) ends. BA comprises nucleotides corresponding to the *rrnB BoxA* (underlined in Table 2) and its left (4 nt) and right (7 nt) flanks. The duplex was cloned into the *Bam*HI site of each pSD construct (see above) to generate plasmids bearing both orientations of the insert, which were selected by restriction analysis and checked over by plasmid sequencing. The final constructs were named as pBASD10, pCompBASD10, and so forth, and the corresponding ENSO strains obtained by homologous recombination as BASD10, CompBASD10, and so forth.

To insert mutations into the *BoxA* sequence of pBASD10, the two-step PCR technique was used. At the first step, two PCR fragments were obtained on pBASD10 as a template with two pairs of primers: UPlac–comp_mutBA and mutBA–DSlac (Table 2). At the second step, the two PCR products were mixed and amplified in the presence of UPlac and DSlac. Finally, the resulting fragment was treated with *Bam*HI and *Hin*dIII and cloned in pEMBLΔ46 to create pMutBASD10. The corresponding ENSO strain obtained by homologous recombination as described above was named MutBASD10.

TABLE 2. List of oligonucleotides.

Name	Sequence (5′ → 3′)
sd3	GATCCA**AAGGAGGTGA**AAAAAC**ATG**AAACAA[a]
sd4	AGCTTTGTTTCATGTTTTTTCACCTCCTTTG
sd5	GATCCA**AAGGAGGT**AAAAAC**ATG**AAACAA[a]
sd6	AGCTTTGTTTCATGTTTTTACCTCCTTTG
sd7	GATCCA**AAGGAG**AAAAAC**ATG**AAACAA[a]
sd8	AGCTTTGTTTCATGTTTTTCTCCTTTG
BA	GATCCACTGCTCTTTAACAATTTATCA[b]
compBA	GATCTGATAAATTGTTAAAGAGCAGTG
mutBA	GATCCACTGgTCTgTAACgAgTTATCAG[c]
comp_mutBA	CTGATAACTCGTTACAGACCAGTGGATC
T7OPlac	TGTAATACGACTCACTATAGGG*AATTGTGAGCGG*[d]
DSlac	GGCGATTAAGTTGGGTAACGCCAGGG[e]
UPlac	GTTAGCTCACTCATTAGGCACCCC[f]

[a]The SD sequences and the start codon are in bold.
[b]The sequence of the *rrnB BoxA* is underlined.
[c]Changes introduced in the *BoxA* sequence are shown by lowercase letters.
[d]The beginning of the genuine *lac*-transcript is italicized.
[e]The primer is complementary to the region (+57 to +82) of the genuine *lacZ* mRNA (+1 is the A of the *lacZ* AUG start codon).
[f]The primer covers the positions from −64 to −41 with respect to the transcriptional start of the *lac*-operon.

Growth of cells and β-galactosidase assay

Cell growth and β-galactosidase assay were performed as described previously (Boni et al., 2000), with minor modifications. Cells were harvested in the exponential phase ($A_{600} \approx$ 0.4–0.6) after at least four generations of balanced growth in LB medium (5 mL) supplemented with IPTG (0.2 mM). Cell pellets obtained by low speed centrifugation at 4 °C were resuspended in 200 μL of chilled PBS buffer containing lysozyme (200 μg/mL) and then subjected to a repeated thawing-freezing procedure. All β-galactosidase activities measured in clarified cell lysates according to Miller (1972) are expressed in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram of total soluble cell proteins.

Toeprinting assay

DNA templates for RNA synthesis in vitro were obtained by PCR, where pSD or pBASD were used as templates, and T7OPlac and DSlac as primers. T7OPlac comprised T7 promoter and *lac*-operator sequences, and DSlac annealed to the *lac* region +57 to +82, wherein position +1 defines the A in the genuine *lacZ* start codon (Table 2). The resulting amplified fragments were used directly for synthesis of RNA with T7 RNA polymerase according to protocols and with the reagents of Promega. The RNAs obtained covered the full 5′ UTR of corresponding mRNAs synthesized in vivo in AKSD10, AKSD6, and BASD10. Extension inhibition analysis of 30S initiation complex formation (Hartz et al., 1988) was performed essentially as described (Boni et al., 1991). The binding buffer for preparation of toeprinting probes was 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM NH_4Cl, 6 mM 2-mercaptoethanol. Each reaction (10 μL) contained ~0.4–0.5 pmol RNA transcript annealed with the 5′-labeled DSlac, 4 pmol of 30S (native or S1-depleted; see Boni et al., 1991, 2001) and 20 pmol of *E. coli* uncharged initiator tRNA, if indicated. Probes were incubated at 37 °C for 10 min and then analyzed by primer extension with AMV reverse transcriptase (Promega).

Toeprinting on the *thrA* mRNAs (with and without an oligoU stretch) was performed in the same way, but the templates for RNA synthesis in vitro were obtained by recloning the *Bam*HI-*Hin*dIII fragments from the corresponding pEMBL derivatives (Boni et al., 2000) into pSP73/*Bgl*II-*Hin*dIII under the control of SP6 promoter. The resulting plasmids were linearized by *Hpa*I downstream from the T7 promoter to serve as templates for transcription with the SP6 RNA-polymerase, and synthesized RNAs were used in a toeprinting assay with the 5′-labeled standard T7 promoter primer.

Gel-mobility shift assay

To prepare high specific activity RNA probes, DNA templates were obtained by PCR with T7OPlac and sd4 as primers (Table 2), and pBASD10, pCompBASD10, and pMutBASD10 as templates. The purified amplified fragments were directly used for run-off transcription with T7 RNA polymerase in the presence of [^{32}P]αUTP (3,000 Ci/mmol; Amersham). To obtain the control RNA lacking any insert, the PCR fragment for RNA synthesis was obtained with pSD10 as a template and T7OPlac and DSlac as primers. The alteration of the downstream PCR primer was done to show that affinity of the RNA to S1 is dependent mainly on the leader sequence and not determined by the RNA length as such. The labeled RNA transcripts (4–5 nM) were incubated for 15 min at 37 °C in the binding buffer with free S1 taken at gradually decreased concentration, then chilled on ice and separated on a nondenaturing 8% polyacrylamide gel at room temperature. Free S1 was prepared using polyU-Sepharose as described (Subramanian, 1983).

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REFERENCES

Balakin AG, Bogdanova SL, Skripkin EA. 1992. mRNA containing an extended Shine–Dalgarno sequence is translated independently of ribosomal protein S1. *Biochem Mol Biol 27*:117–129.

Boni IV, Artamonova VS, Dreyfus M. 2000. The last RNA-binding repeat of the *Escherichia coli* ribosomal protein S1 is specifically involved in autogenous control. *J Bacteriol 182*:5872–5879.

Boni IV, Artamonova VS, Tzareva NV, Dreyfus M. 2001. Non-canonical mechanism for translational control in bacteria: Synthesis of ribosomal protein S1. *EMBO J 20*:4222–4232.

Boni IV, Issaeva DM, Musychenko ML, Tzareva NV. 1991. Ribosome-messenger recognition: mRNA target sites for ribosomal protein S1. *Nucleic Acids Res 19*:155–162.

Calogero RA, Pon CL, Canonaco MA, Gualerzi CO. 1988. Selection of the mRNA translation initiation region by *Escherichia coli* ribosomes. *Proc Natl Acad Sci USA 85*:6427–6431.

de Boer HA, Comstock LJ, Hui A, Wong E, Vasser M. 1983. A hybrid promoter and portable Shine–Dalgarno regions of *Escherichia coli*. *Biochem Soc Symp 48*:233–244.

Dreyfus M. 1988. What constitutes the signal for the initiation of protein synthesis on *Escherichia coli* mRNAs? *J Mol Biol 204*: 79–94.

Ellinger T, Behnke D, Bujard H, Gralla JD. 1994. Stalling of *Escherichia coli* RNA polymerase in the +6 to +12 region in vivo is associated with tight binding to consensus promoter elements. *J Mol Biol 239*:455–465.

Etchegaray J-P, Inouye M. 1999. Translational enhancement by an element downstream of the initiation codon in *Escherichia coli*. *J Biol Chem 274*:10079–10085.

Fargo DC, Boynton JE, Gillham NW. 1999. Mutations altering the predicted secondary structure of a chloroplast 5′ untranslated region affect its physical and biochemical properties as well as its ability to promote translation of reporter mRNAs both in the *Chlamidomonas reinhardtii* chloroplasts and in *Escherichia coli*. *Mol Cell Biol 19*:6980–6990.

Fargo DC, Zhang M, Gillham NW, Boynton JE. 1998. Shine–Dalgarno-like sequences are not required for translation of chloroplast mRNAs in *Chlamidomonas reinhardtii* chloroplasts or in *Escherichia coli*. *Mol Gen Genet 257*:271–282.

Farwell MA, Roberts MW, Rabinowitz JC. 1992. The effect of ribosomal protein S1 from *Escherichia coli* and *Micrococcus luteus* on protein synthesis *in vitro* by *E. coli* and *Bacillus subtilis*. *Mol Microbiol 6*:3375–3383.

Fatscher HP, Geisen RM, Fuchs E. 1988. Only one out of three strong ribosomal binding sites of the early region of bacteriophage T7 exhibits high translational efficiency in fragments of about 30 base pairs. *Eur J Biochem 175*:461–465.

Freier SM, Kierzek R, Jaeger JA, Sugimoto N, Caruthers MH, Neilson T, Turner DH. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc Natl Acad Sci USA 83*:9373–9377.

Gallie DR, Kado CI. 1989. A translational enhancer derived from tobacco mosaic virus is functionally equivalent to a Shine–Dalgarno sequence. *Proc Natl Acad Sci USA 86*:129–132.

Gold L. 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. *Annu Rev Biochem* 57:199–233.

Golshani A, Kolev V, AbouHaidar MG, Ivanov IG. 2000. Epsilon as an initiator of translation of CAT mRNA in *Escherichia coli*. *Biochem Biophys Res Commun 273*:528–531.

Gualerzi CO, Pon CL. 1990. Initiation of mRNA translation in prokaryotes. *Biochemistry 29*:5881–5889.

Hartz D, McPheeters DS, Gold L. 1991a. Influence of mRNA deter-

minants on translation initiation in *Escherichia coli*. *J Mol Biol 218*:83–97.
Hartz D, McPheeters DS, Green L, Gold L. 1991b. Detection of *Escherichia coli* ribosome binding at translation initiation sites in the absence of tRNA. *J Mol Biol 218*:99–105.
Hartz D, McPheeters DS, Traut R, Gold L. 1988. Extension inhibition analysis of translation initiation complexes. *Methods Enzymol 164*:419–425.
Herschlag D. 1995. RNA chaperons and the RNA folding problem. *J Biol Chem 270*:20871–20874.
Hui A, de Boer H. 1987. Specialized ribosomes system: Preferential translation of a single mRNA species by a subpopulation of mutated ribosomes in *Escherichia coli. Proc Natl Acad Sci USA 84*:4762–4766.
Jackson RJ. 2000. A comparative view of initiation site selection mechanisms. In: Sonnenberg N, Hershey JWB, Mathews MB, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 127–183.
Jacobs WF, Santer M, Dahlberg AE. 1987. A single base change in the Shine–Dalgarno region of 16S rRNA of *Escherichia coli* affects translation of many proteins. *Proc Natl Acad Sci USA 84*:4757–4761.
Loechel S, Inamine JM, Hu PC. 1991. A novel translation initiation region from *Mycoplasma genitalium* that functions in *Escherichia coli. Nucleic Acids Res 19*:6905–6911.
McCarthy JEG, Brimacombe R. 1994. Prokaryotic translation: The interactive pathway leading to initiation. *Trends Genet 10*: 402–407.
McCarthy JEG, Gualerzi C. 1990. Translational control of prokaryotic gene expression. *Trends Genet 6*:78–85.
McCarthy JEG, Shairer HU, Sebald W. 1985. Translation initiation frequency of *atp* genes from *Escherichia coli*: Identification of an intercistronic sequence that enhances translation. *EMBO J 4*:519–526.
Melançon P, Leclerc D, Destroismaisons N, Brakier-Gingras L. 1990. The anti-Shine–Dalgarno region in *Escherichia coli* 16S ribosomal RNA is not essential for the correct selection of translational starts. *Biochemistry 29*:3402–3407.
Miller JH. 1972. *Experiments in molecular genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Mogridge J, Greenblatt J. 1998. Specific binding of *Escherichia coli* ribosomal protein S1 to *boxA* transcriptional antiterminator RNA. *J Bacteriol 180*:2248–2252.
Moll I, Huber M, Grill S, Sairafi P, Mueller F, Brimacombe R, Londai P, Blasi U. 2001. Evidence against an interaction between the mRNA downstream box and 16S rRNA in translation initiation. *J Bacteriol 183*:3499–3505.
O'Connor M, Asai T, Squires CL, Dahlberg AE. 1999. Enhancement of translation by the downstream box does not involve base pairing of mRNA with the penultimate stem sequence of 16S rRNA. *Proc Natl Acad Sci USA 96*:8973–8978.
O'Connor M, Dahlberg AE. 2001. Enhancement of translation by the epsilon element is independent of the sequence of the 460 region of 16S rRNA. *Nucleic Acids Res 29*:1420–1425.
Olins PO, Rangwalla SH. 1989. A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the *lacZ* gene in *Escherichia coli*. *J Biol Chem 264*:16973–16976.
Ringquist S, Jones T, Snyder E, Gibson T, Boni I, Gold L. 1995. High-affinity RNA ligands to *Escherichia coli* ribosomes and ribosomal protein S1: Comparison of natural and unnatural binding sites. *Biochemistry 34*:3640–3648.
Ringquist S, Shinedling S, Barrick D, Green L, Binkley J, Stormo GD, Gold L. 1992. Translation initiation in *Escherichia coli*: Sequences within the ribosome-binding site. *Mol Microbiol 6*:1219–1229.
Roberts MW, Rabinowitz JC. 1989. The effects of *Escherichia coli* ribosomal protein S1 on the translational specificity of bacterial ribosomes. *J Biol Chem 264*:2228–2235.
Sacerdot C, Caillet J, Graffe M, Eyermann F, Ehresmann B, Ehresmann C, Springer M, Romby P. 1998. The *Escherichia coli* threonyl-tRNA synthetase gene contains a split ribosomal binding site interrupted by a hairpin structure that is essential for autoregulation. *Mol Microbiol 29*:1077–1090.
Schneider TD, Stormo GD, Gold L, Ehrenfeucht A. 1986. Information content of binding sites on nucleotide sequences. *J Mol Biol 188*:415–431.
Sengupta J, Agrawal RK, Frank J. 2001. Visualization of protein S1 within the 30S ribosomal subunit and its interaction with messenger RNA. *Proc Natl Acad Sci USA 98*:11991–11996.
Shean CS, Gottesman ME. 1992. Translation of the prophage λ cI transcript. *Cell 70*:513–522
Shine J, Dalgarno L. 1974. The 3′-terminal sequence of *E. coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. *Proc Natl Acad Sci USA 71*:1342–1346.
Sørensen MA, Fricke J, Pedersen S. 1998. Ribosomal protein S1 is required for translation of most, if not all, natural mRNAs in *Escherichia coli* in vivo. *J Mol Biol 280*:561–569.
Sprengart ML, Fuchs E, Porter AG. 1996. The downstream box: An efficient and independent translation initiation signal in *Escherichia coli*. *EMBO J 15*:665–674.
Steitz JA, Jakes K. 1975. How ribosomes select initiator regions in mRNA: base pair formation between the 3′ terminus of 16S RNA and the mRNA during initiation of protein synthesis in *E. coli*. *Proc Natl Acad Sci USA 72*:4734–4738.
Subramanian AR. 1983. Structure and functions of ribosomal protein S1. *Prog Nucleic Acid Res Mol Biol 28*:101–142.
Tzareva NV, Makhno VI, Boni IV. 1994. Ribosome-messenger recognition in the absence of the Shine–Dalgarno interactions. *FEBS Lett 337*:189–194.
Van Etten WJ, Janssen GR. 1998. An AUG initiation codon, not codon–anticodon complementarity, is required for the translation of unleadered mRNA in *Escherichia coli*. *Mol Microbiol 27*: 987–1001.
Vogel U, Jensen KF. 1995. Effects of the antiterminator BoxA on transcription elongation kinetics and ppGpp inhibition of transcription elongation in *Escherichia coli*. *J Biol Chem 270*: 18335–18340.
Wilson TMA. 1986. Expression of the large 5′-proximal cistron of tobacco mosaic virus by 70S ribosomes during cotranslational disassembly in a prokaryotic cell-free system. *Virology 152*: 277–279.
Wu C-J, Janssen GR. 1997. Expression of a streptomycete leaderless mRNA encoding chloramphenicol acetyltransferase in *Escherichia coli*. *J Bacteriol 179*:6824–6830.
Yarchuk O, Jacques N, Guillerez J, Dreyfus M. 1992. Interdependence of translation, transcription and mRNA degradation in the *lacZ* gene. *J Mol Biol 226*:581–596.
Zhang J, Deutscher MP. 1992. A uridine-rich sequence required for translation of prokaryotic mRNA. *Proc Natl Acad Sci USA 89*: 2605–2609.