Evidence against an Interaction between the mRNA Downstream Box and 16S rRNA in Translation Initiation

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The downstream box (db) located 8 to 13 nucleotides (nt) downstream of the start codon has been originally proposed to base pair with the anti-downstream box (adb) spanning nt 1469 to 1483 within helix 44 of the 16S rRNA (Fig. 1A; 37). The db element was identified first in the highly expressed genes 0.3 and 10 of *Escherichia coli* phage T7 and has been reported to stimulate translation per se or in combination with the Shine-Dalgarno (SD) sequence (37, 38). Since the original proposal, there has been much publicity in favor of the proposed db-adb interaction (3, 6, 7, 8, 9, 16, 21, 23, 26, 33, 43, 44). In some cases, the importance of the db-adb interaction in translation initiation was proposed solely based on computer analysis in the complete absence of any experimental data. The supporting experimental evidence for the db-adb base pairing rests entirely on manipulations of mRNAs containing a putative db and the observations that, in general, increases in complementarity of the db with the adb resulted in an increased expression of the db-containing helix 44 that disrupt the helical continuity of the ada fragment. These effects could be reversed upon introduction of compensatory mutations that restored base pairing within the helix (10). These studies indicated that a stable helix rather than a particular primary sequence is important for ribosome function. Recently, O’Connor et al. (28) performed a landmark experiment by reversing all 12 bp of the stem containing the putative adb, thereby creating a mutant 16S rRNA with a radically altered base pairing potential. This 16S rRNA allele with the adb-flip has been expressed in an *E. coli* strain in which all of the seven *rrn* operons had been deleted (2). The expression rates of several previously described db-containing reporter constructs were found to be indistinguishable in both the adb-flip mutant and the isogenic wild-type (wt) strain. These genetic studies showed that any db-associated enhancer activity does not involve db-adb base pairing (28). The db-adb interaction has been suggested to be instrumental for the translation of *E. coli cspA* mRNA during cold shock, and it seemed conceivable that the adb is particularly exposed in cold-shocked ribosomes (8, 21). Using the same approach as O’Connor et al. (28), La Teana et al. (18) have recently shown that 30S db-flip mutants translated the cspA mRNA with the same efficiency as wt ribosomes under cold shock as well as under non-cold shock conditions, suggesting again that the db-adb base pairing is irrelevant.

It has been suggested that the db serves as an independent translation initiation signal (38). In another report, it has been suggested that the db-adb and the SD/anti-SD (aSD) interactions act synergistically to enhance translation initiation (7).
However, no direct in vitro experiments have been performed to address the question of whether an mRNA with an optimal fit to the adb has an increased affinity for the 3OS subunit as would be expected regardless of whether the db provides per se a ribosomal recruitment signal, whether it acts in concert with the SD sequence, or whether it acts transiently to increase the concentration of the start codon close to the decoding center (39). Here, we present genetic and biochemical studies as well as topographical information on the ribosome which, taken together with previously performed genetic studies (18, 28), add strong evidence against the db-adb interaction.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *E. coli* strains MS59a (31) and MC4100F- (40) as well as the *Bacillus subtilis* strain 168 (1) have been described. They were grown in Luria-Bertani medium (20) supplemented with ampicillin (100 μg/ml) where appropriate to maintain selection of plasmids. Growth of the liquid cultures was monitored photometrically by measuring the optical density at a wavelength of 600 nm (OD600).

Plasmid pAXL7 harbors the full-length cl gene under transcriptional control of a T7 promoter (41).

**Construction of plasmids used in this study.** Plasmids pRB381-1 and pRB381-3 are derivatives of plasmid pRB381 (4). They were constructed as follows. Plasmid pMCCcl(63) (31) was digested with XhoI and SmaI, and the fragment was inserted into the corresponding sites of plasmid pUC18 (19). The resulting pUC18 derivative was then cleaved with XhoI and BamHI, and the fragment containing the λ promotore (from nt 2 to 40) as well as the *E. coli* 16S rRNA and spatial localization of the adb in the 3OS subunit of *T. thermophilus.* (A) Helices 44 and 45 of the *E. coli* 16S rRNA are enlarged. The adb sequence shown boxed (nt 1469 to 1483) in helix 44 has been suggested to base pair with the db in mRNA, i.e., with mRNA downstream of the start codon. (B) Placement of the mRNA (nt −6 to +12; green tube) in the *T. thermophilus* 16S rRNA based on cross-linking studies (35). The positions of the aSD sequence and the adb in the 16S rRNA are shown. The aSD sequence and the putative adb region are depicted in magenta. The mRNA is shown as a green tube from nt −6 to +12 with regard to the A (+1) of the start codon. The P-site 16S rRNA is shown in orange. (C) Stereoview of the structure shown in panel B added to the contour of the 3OS subunit (see text).

**β-galactosidase assays.** The β-galactosidase activities were determined as described by Miller (20). Triplicate aliquots were taken of each culture at an OD600 of 0.8.

**In vitro translation.** Full-length *Lactococcus lactis* phage r1r pro mRNA was obtained as described previously (22) except that the reverse “PCR-oligonucleotide” was complementary to a region downstream of the pro stop codon. For full-length synthesis of cl mRNA, plasmid pAXL7 was cleaved with PstII. The PCR fragment and the linearized plasmid served as templates for in vitro transcription reactions with T7 RNA polymerase. The in vitro translation reactions with *Salibolodus solitarius* extracts were performed as described by Condor et al. (5). The samples contained the following in a final volume of 50 μl: 10 mM KCl, 20 mM Tris-HCl (pH 7.0), 18 mM MgOAc, 7 mM β-mercaptoethanol, 3 mM ATP, 1 mM GTP, 5 μg of bulk *S. solitarius* mRNA, 20 μM amino acids (except for methionine), 2 μM of [35S]methionine (1,200 Ci/mmol), 20 μl of *S. solitarius* S30 extract (precultivated for 10 min at 73°C), and 10 pmol of mRNA. The samples were incubated at 73°C for 40 min. The labeled proteins were separated on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel. The gel was dried under vacuum and exposed to an X-ray film.
Computer graphics and modeling of helix 44 on the 30S subunit. All of the relevant procedures for modeling of helix 44 on the 30S subunit have been described in detail by Mueller and Brimacombe (24, 25).

RESULTS AND DISCUSSION

Spatial organization of mRNA-db and adb on the 30S subunit. The recently published crystal structure of Thermus thermophilus 16S rRNA (42) is shown in Fig. 1B. Nucleotides in the E. coli 16S rRNA which have been cross-linked to downstream positions (+2, +4, etc.) in the mRNA (35) are highlighted in red as CPK models. It should be noted that attempts to cross-link the putative adb to mRNA have failed (R. Brimacombe, unpublished results).

In Fig. 1C, the structure shown in Fig. 1B was added to the contour of the 30S subunit obtained by electron cryomicroscopy (24). The stereo views show that helix 44 is far from the decoding site and runs down to the bottom of the interface site of the E. coli 30S subunit with the adb situated approximately in the middle of the body. In the translation initiation complex, the mRNA cross-links are grouped around the “hole,” and part of the putative db on mRNA would pass through the hole. Thus, the putative db is exposed to the solvent site and the whole shoulder of the body of the 30S subunit is situated between the putative db on the mRNA and the adb in helix 44 (Fig. 1C). Taken together with other topographical information on the 30S ribosome (11, 34, 42), models where the start codon can be placed in the ribosomal P site while the adjacent db interacts simultaneously with the adb (38) or where the db acts synergistically with the SD/aSD interaction (7) are not tenable. These topographical constraints are supported by previous chemical probing experiments which showed that the putative db on λ cI mRNA is not protected from modifying reagents in 70S initiation complexes (31).

An optimal db does not result in 30S subunit binding to leaderless λ cI mRNA. Although the topography of the ribosome apparently excludes that the db can mechanistically substitute for the SD sequence, it seemed worth testing the hypothesis that the db-adb interaction could contribute to initial interactions between the 30S subunit and mRNA prior to formation of the translation initiation complex (39). We have argued that a contribution of the putative db-adb interaction in ribosome mRNA binding may be best assessed in the absence of an SD/aSD interaction (31). Therefore, the relative affinities of 30S subunits were determined for leaderless λ cI wt mRNA and for a derivative thereof containing an “optimized db” as well as for a cI mRNA derivative with a 5′-terminal extension containing an SD sequence (Fig. 2). λ cI34 RNA contains the first 34 nt, and 8 consecutive nucleotides are complementary to the putative adb (Fig. 2). The λ cIOptdb comprises 33 nt, of which nt 1 to 15 are fully complementary to the putative adb (Fig. 2). Both were added to a 10-fold molar excess of 30S subunits (conditions which reflect the relative affinity constants of the corresponding complexes). As shown in Fig. 2, only 0.37% of cI34 mRNA and 0.30% of cIOptdb mRNA formed a binary complex with 30S subunits. When the cI34SD mRNA (Fig. 2) was used as a binding substrate, approximately 46% of this mRNA formed a binary complex with ribosomes. Thus, in contrast to the SD/aSD interaction, an optimized base pairing potential did not increase the affinity of ribosomes for the cIOptdb mRNA. In other words, an optimized db cannot provide sufficient interactions with the 30S ribosome to stimulate initial binding of the mRNA to the ribosome. The addition of a 2-fold molar excess of fMet-tRNAfMet over ribosomes increased the amount of bound cI34 mRNA approximately 4-fold, that of cIOptdb mRNA 3-fold, and that of cI34SD mRNA 1.6-fold. Since the topography of the 30S subunits (Fig.
optimized db did not increase translation of the codon (see Materials and Methods). As shown in Fig. 3B, the transcription started at the adenosine of the AUG initiating abutted the start codon of the

The failure of 30S ribosomes to form a binary complex with λ cI50db mRNA is consistent with kinetic toeprint experiments (31) and with studies performed by La Teana et al. (18), who have recently shown that the adb is not accessible to a cDNA oligonucleotide while the anti-SD sequence, which served as a control, was fully accessible. Ever since the db-adb base pairing was first proposed, it has been perplexing that it would require extensive unwinding of the 16S rRNA stem comprising the adb. Again, La Teana et al. (18) have shown, using chemical probing, that the adb is in a double-stranded conformation and therefore is not available for base pairing.

An optimized db does not enhance translation of a leaderless λ cI-lacZ construct. We have previously shown that elimination of 5 nt of the putative λ cI db did not affect the translational efficiency of the corresponding cI-lacZ construct (13, 31). However, this work has been criticized in that alternative db stretches would have been recreated (6, 39). Therefore, we next addressed the question whether an optimized db rather than a partial deletion of the db in λ mRNA would affect the translation of a reporter construct in vivo. Two different plasmids were used. Plasmid pRB381-1 contains the first 63 codons of the wt cI gene fused to the lacZ gene, whereas in pRB381-3, the immediate coding region of the cI-lacZ fusion gene was altered such that a consecutive stretch of 10 nt, including three G:C pairs, showed complementarity to the adb of B. subtilis (Fig. 4A). Therefore, we asked whether a cI-lacZ reporter gene carried by plasmid pRB381c1 (see Materials and Methods) is expressed in B. subtilis. The cI63-lacZ fusion was translated in B. subtilis despite the insignificant complementarity between the putative db of cI and the putative adb of B. subtilis. The absolute expression levels of the reporter construct may vary in E. coli and B. subtilis due to differential transcriptional, posttranscriptional, or translational regulatory events. It is therefore not possible to compare the translational efficiencies in both organisms directly. However, the cI63-lacZ fusion is translated in B. subtilis at a rather high level (1,050 ± 45 Miller units), which would not be expected if the db-adb base pairing was essential. It seems worth noting that the coding sequences from +4 to +33 of all B. subtilis genes did not reveal a statistically significant motif, i.e., a db (32). Moreover, these authors randomized the sequences of the putative db region and observed that 34% of the sequences showed a db with eight matches while 9% had one with nine matches, which led them to conclude that the db “patterns” are statistically irrelevant.

We have recently shown that the λ cI mRNA is faithfully translated in an in vitro translation system derived from the...
archaeon *S. solfataricus* (12) although the region of helix 44 comprising the putative adb is highly dissimilar in *E. coli* and *S. solfataricus* (Fig. 4B). To extend the heterologous expression studies, the translation rates of both λ cI mRNA and *L. lactis* phage r1t rro mRNA encoding the phage repressor (27) were compared in the *S. solfataricus* in vitro translation system. As for *E. coli*, the putative adb of *L. lactis* is highly dissimilar compared to that of *S. solfataricus*. In the in vitro translation system was programmed with equimolar amounts of λ cI mRNA and rro mRNA. Despite the differences in the respective adb sequences of *E. coli*, *L. lactis*, and *S. solfataricus* (Fig. 4B), both mRNAs were expressed with approximately the same efficiency (Fig. 4C). At first glance, this may be fortuitous. However, in light of the current view on translation initiation of leaderless mRNAs, it may not be surprising. Grill et al. (12) have recently shown that the start codon is the only constant and necessary element of the leaderless λ cI mRNA that is recognized by the ribosome. Moreover, since translation initiation factor 2 (IF2) selectively stimulated translation of a leaderless mRNA in vitro as well as in vivo, it has been concluded that the 5′-terminal start codon of leaderless mRNAs is recognized by a 30S-initiator-tRNA-IF2 complex, an intermediate equivalent to that obligatorily formed during translation initiation in eukaryotes (12). A homologue of both the eukaryal IF2 and the bacterial type IF2 is present in *S. solfataricus* (P. Londei, unpublished results). Therefore, the translation initiation pathway proposed for leaderless mRNAs in *E. coli* (12) could operate in this organism as well, which would explain the similar expression rates of both the λ cI and r1t rro mRNA observed with the *S. solfataricus* in vitro translation system. In agreement with these studies, Condo et al. (5) have shown that two different open reading frames of *S. solfataricus* were translated in the homologous in vitro translation system upon removal of the upstream leader sequence, including an SD sequence, despite the absence of any sequence motif in the 5′ initial coding region with complementarity to the corresponding adb. In addition, we have previously demonstrated the heterologous expression of cI mRNA in a *Bacillus stearothermophilus* in vitro translation system (41) and of cI mRNA in a mammalian reticulocyte lysate (12), as well as in vitro ternary complex formation on rro mRNA with *E. coli* ribosomes (22). Again, the putative adb sequences are not conserved in these organisms. Our expression studies in different organisms are in agreement with the genetic studies of Firpo and Dahlberg (10) and of O’Connor et al. (28), who demonstrated that the continuity and stability of helix 44 is important for ribosome function rather than its primary sequence.

**Conclusions.** In summary, there is neither biochemical nor genetic evidence for the proposed adb-adb interaction in terms of ribosome recruitment via rRNA-mRNA contacts during translation initiation. The topography of the 30S subunit can explain why the db-adb interaction cannot act simultaneously with the SD/aSD interaction. In addition, the sequestration of the putative adb within helix 44 readily explains why ribosomes do not form a binary complex with an mRNA comprising an optimal-fit db or with a db-oligonucleotide. While these and other data (18, 28, 31) argue against the molecular mechanism originally proposed for the db (37–39), they do not invalidate studies wherein the db has been suggested to enhance translation initiation in particular mRNAs. Therefore, other models to explain the stimulating effects of this downstream enhancer should be tested.

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