

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

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Based on the complementarity of the initial coding region (downstream box [db]) of several bacterial and phage mRNAs to bases 1469 to 1483 in helix 44 of 16S rRNA (anti-downstream box [adb]), it has been proposed that db-adb base pairing enhances translation in a way that is similar to that of the Shine-Dalgarno (SD)/anti-Shine-Dalgarno (aSD) interaction. Computer modeling of helix 44 on the 30S subunit shows that the topography of the 30S ribosome does not allow a simultaneous db-adb interaction and placement of the initiation codon in the ribosomal P site. Thus, the db-adb interaction cannot substitute for the SD-aSD interaction in translation initiation. We have always argued that any contribution of the db-adb interaction should be most apparent on mRNAs devoid of an SD sequence. Here, we show that 30S ribosomes do not bind to leaderless mRNA in the absence of initiator tRNA, even when the initial coding region shows a 15-nucleotide complementarity (optimal fit) with the putative adb. In addition, an optimized db did not affect the translational efficiency of a leaderless λ *cI-lacZ* reporter construct. Thus, the db-adb interaction can hardly serve as an initial recruitment signal for ribosomes. Moreover, we show that different leaderless mRNAs are translated in heterologous systems although the sequence of the putative adb's within helix 44 of the 30S subunits of the corresponding bacteria differ largely. Taken our data together with those of others (M. O'Connor, T. Asai, C. L. Squires, and A. E. Dahlberg, *Proc. Natl. Acad. Sci. USA* 96:8973–8978, 1999; A. La Teana, A. Brandi, M. O'Connor, S. Freddi, and C. L. Pon, *RNA* 6:1393–1402, 2000), we conclude that the db does not base pair with the adb.

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, 36, 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 and decreases in the db-adb complementarity had the corresponding downward effects on expression (9, 36, 38).

Biochemical evidence for the db-adb interaction is lacking. Chemical protection studies on λ *cI* mRNA-70S initiation complexes (31) and on phage T4 gene 32 mRNA-30S and -70S complexes (15) have failed to show protection of the putative db. Mutational studies revealed that alterations on either side

of the adb-containing helix 44 that disrupt the helical continuity had deleterious effects on ribosome function. 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 *rrm* 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).

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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 30S 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 MS59 λ (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 μ g/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 *cI* 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 pMCcI(63) (31) was digested with *Xba*I and *Sma*I, and the fragment was inserted into the corresponding sites of plasmid pUC18 (19). The resulting pUC18 derivative was then cleaved with *Xba*I and *Bam*HI, and the fragment containing the λ p_{RM} promoter and the first 63 codons of the *cI* gene was inserted into the corresponding sites of pRB381, resulting in pRB381-1.

The putative *db* in λ *cI* was optimized in plasmid pRB381-3 by using the PCR-based site-directed mutagenesis procedure described by Hall and Emery (14). Briefly, the immediate 5'-coding region of the *cI* gene was modified as shown in Fig. 3A, using complementary oligonucleotides comprising this region in conjunction with biotinylated oligonucleotides for the upstream and downstream region of the part of the *cI* gene present in plasmid pMCcI(63) (31). After hybridization of the two strands and the fill-in reaction with T4 DNA polymerase, a PCR with nonbiotinylated oligonucleotides was performed. The resulting fragment was cleaved with *Xba*I and *Bam*HI and cloned into the corresponding sites of the vector pRB381, resulting in plasmid pRB381-3. The plasmids pRB381-1 and pRB381-3 are isogenic except for the initial coding region of the *cI* gene (optimized *db**; see Fig. 3A).

Plasmid pRB381cI is a derivative of the *E. coli*-*B. subtilis* shuttle vector pRB381 and was constructed as follows. First, a PCR fragment containing the *lac* promoter (from nt -60 to +1) from plasmid pUHE21-2 (17) and the first 189 nt of the λ *cI* gene were inserted into the *Xba*I-*Sma*I sites of plasmid pKT35 (30). In the resulting plasmid, pKTplaccI, the *lac* promoter abutted the start codon of the *cI* gene via an *Nco*I site, which ensured that transcription of *cI* mRNA starts at the adenosine of the start codon. Plasmid pKTplaccI was then used as a template with an *Xba*I forward primer and a *Bam*HI reverse primer. The PCR product was cleaved with *Xba*I and *Bam*HI, and the fragment was inserted into the same sites of pRB381. In the resulting plasmid pRB381cI, the *cI*-*lacZ* mRNA, which contains the first 63 codons of the *cI* gene, is transcribed from the modified *lacpo*. The transcript starts with the A of the initiating codon of the *cI* gene. All DNA manipulations were verified by sequencing.

Filter-binding assay. Filter-binding assays were performed using a Schleicher and Schuell SRC 072/0 Minifold II Slot Blot apparatus. The *cI*34 mRNA, *cI*^{Optdb} mRNA, and *cI*34SD mRNA (Fig. 2) were obtained by hybridization of cDNA nucleotides containing the T7 ϕ 10 promoter and either the first 34 bp (*cI*34 and *cI*34SD mRNAs, including the 5' extension) of the *cI* gene or the first 33 bp of *cI* with the optimized *db* (*cI*^{Optdb} mRNA) and subsequent T7 RNA polymerase-directed mRNA synthesis. All mRNAs were labeled with [α -³²P]CTP. In a reaction volume of 50 μ l, 0.5 pmol of ³²P-labeled mRNA was incubated with 30S ribosomes at a molar ratio of 1:10 (Fig. 2). fMet-tRNA^{Met} was added in a twofold excess over 30S ribosomes. The reaction mixtures were incubated for 10 min at 37°C. Samples were added to the filter under vacuum and washed with 25 volumes of VD+ reaction buffer as previously described (41). The filters were exposed to a Molecular Dynamics PhosphorImager screen for quantitation of the retained mRNA. The total counts per minute values were corrected for the number of C's in each mRNA.

A

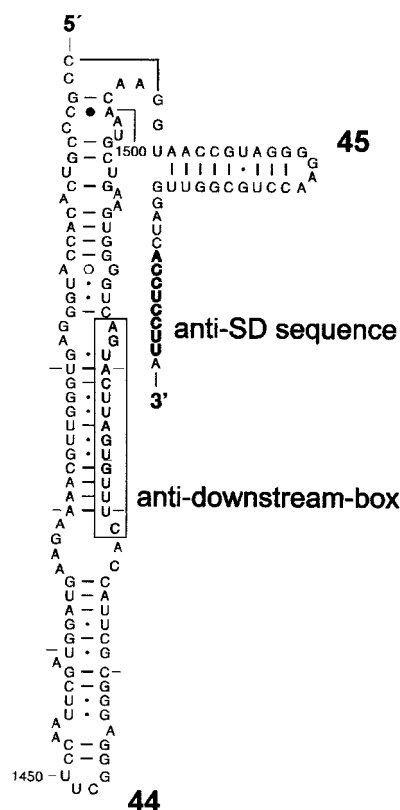
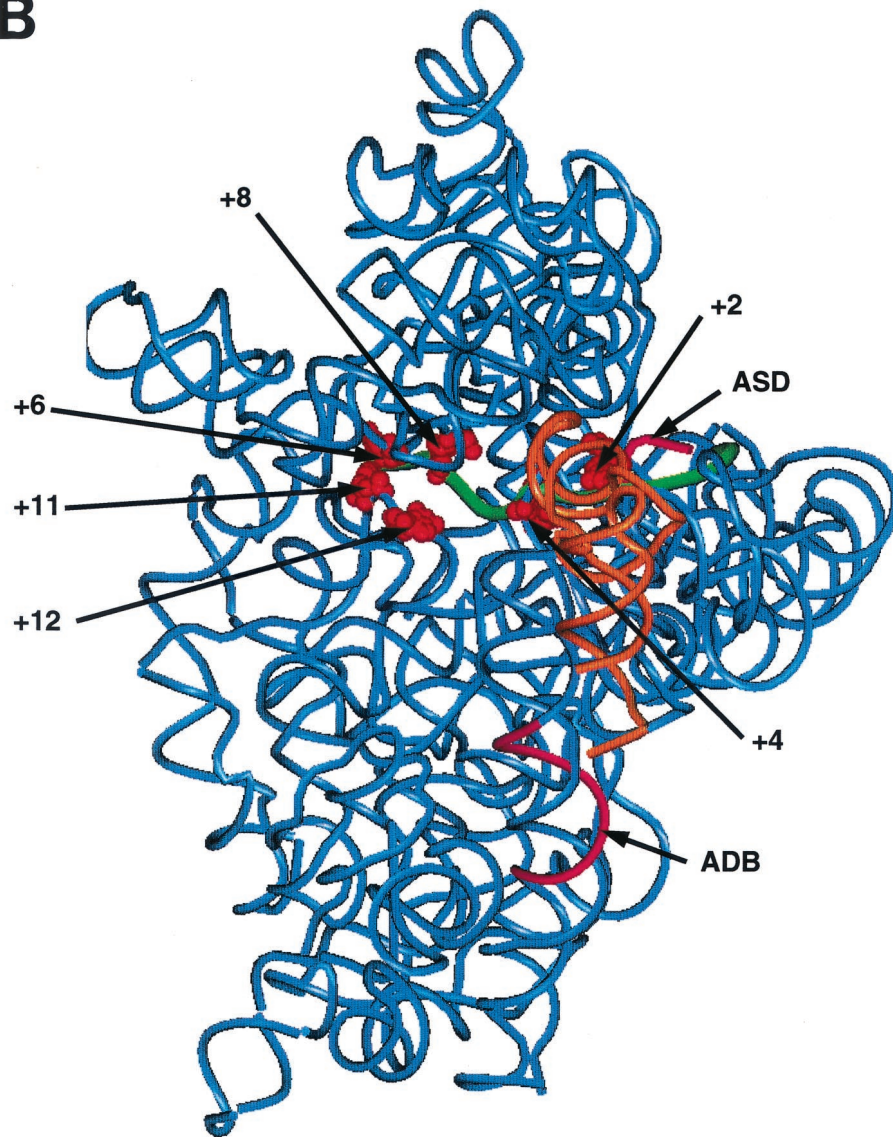


FIG. 1. Localization of the putative *adb* in the penultimate stem of *E. coli* 16S rRNA and spatial localization of the *adb* in the 30S subunit of *T. thermophilus*. (A) Helices 44 and 45 of the *E. coli* 16S rRNA are enlarged. The *adb* sequence shown boxed (nt 1469 to 1483) in helix 44 has been suggested to base pair with the *db* in mRNA, i.e., with mRNA sequences 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 tRNA is shown in orange. (C) Stereoview of the structure shown in panel B added to the contour of the 30S 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 r1t *ro* mRNA was obtained as described previously (22) except that the reverse "PCR-oligonucleotide" was complementary to a region downstream of the *ro* stop codon. For full-length synthesis of *cI* mRNA, plasmid pAXL7 was cleaved with *Pvu*II. 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 *Sulfolobus solfataricus* extracts were performed as described by Condo et al. (5). The samples contained the following in a final volume of 50 μ l: 10 mM KCl, 20 mM Tris-HCl (pH 7.0), 18 mM MgOAc, 7 mM β -mercaptoethanol, 3 mM ATP, 1 mM GTP, 5 μ g of bulk *S. solfataricus* tRNA, 20 μ M amino acids (except for methionine), 2 μ l of [³⁵S]methionine (1,200 Ci/mmol), 20 μ l of *S. solfataricus* S30 extract (preincubated 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.

B



C

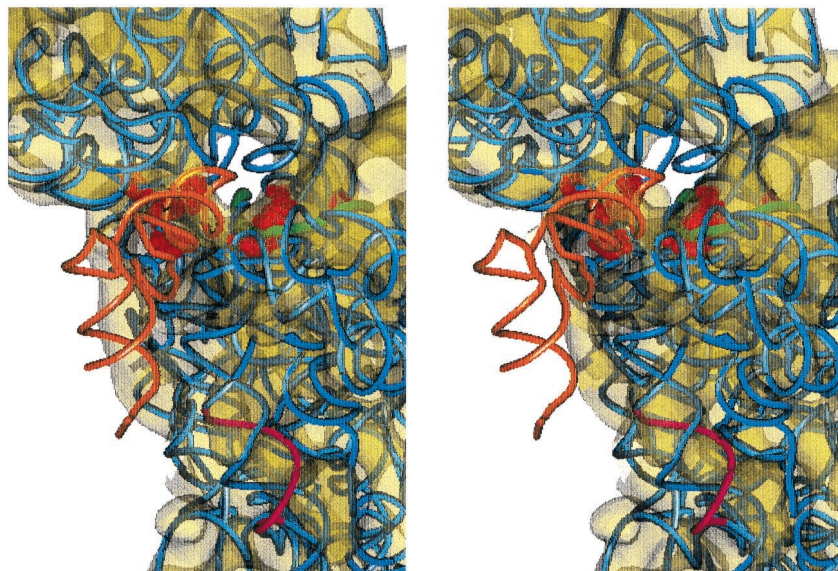


FIG. 1—Continued.

		cI34 mRNA	cI ^{Optdb} mRNA	cI34SD mRNA
	ratio of 30S ribosomes/mRNA	Percentage of input mRNA retained with 30S ribosomes	Percentage of input mRNA retained with 30S ribosomes	Percentage of input mRNA retained with 30S ribosomes
Binary complex	10:1	0.37±0.1	0.30±0.12	45.80±1.5
Ternary complex	10:1	1.54±1.15	0.92±0.11	70.33±1.9
cI34 mRNA:	5' - <u>AUGAGCACAAA</u> AAAGAAACCAUUAACACAAGAGC -3'			
cI ^{Optdb} mRNA:	5' - <u>AUGAAUCACAAAGUG</u> CCAUUAACACAAGAGC -3'			
cI34SD mRNA:	5' - GUCUAGAAAUAUUUUGUUUAACUUUA <u>AAGAAGAGAU</u> AUACAU <u>AUGAGCACAAA</u> AAAGAAACCAUUAACACAAGAGC -3'			
16S rRNA adb:	3' - AGU <u>ACU</u> AGUGUUUCAC -5			
	1483	1467		

FIG. 2. The cI34, cI^{Optdb}, and cI34SD mRNAs are depicted. The putative db sequences as well as the putative adb in *E. coli* 16S rRNA are shown in bold. The start codons and the SD sequence in cI34SD mRNA are underlined. The total counts per minute values corrected for the number of C's in each mRNA were set at 100% (mRNA input). Ternary complexes were allowed to form in the presence of a twofold molar excess of fMet-tRNA_f^{Met} over ribosomes.

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 mechanically 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 cI^{Optdb} 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 cI^{Optdb} 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 optimal base pairing potential did not increase the affinity of ribosomes for the cI^{Optdb} 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-tRNA_f^{Met} over ribosomes increased the amount of bound cI34 mRNA approximately 4-fold, that of cI^{Optdb} mRNA 3-fold, and that of cI34SD mRNA 1.6-fold. Since the topography of the 30S subunits (Fig.

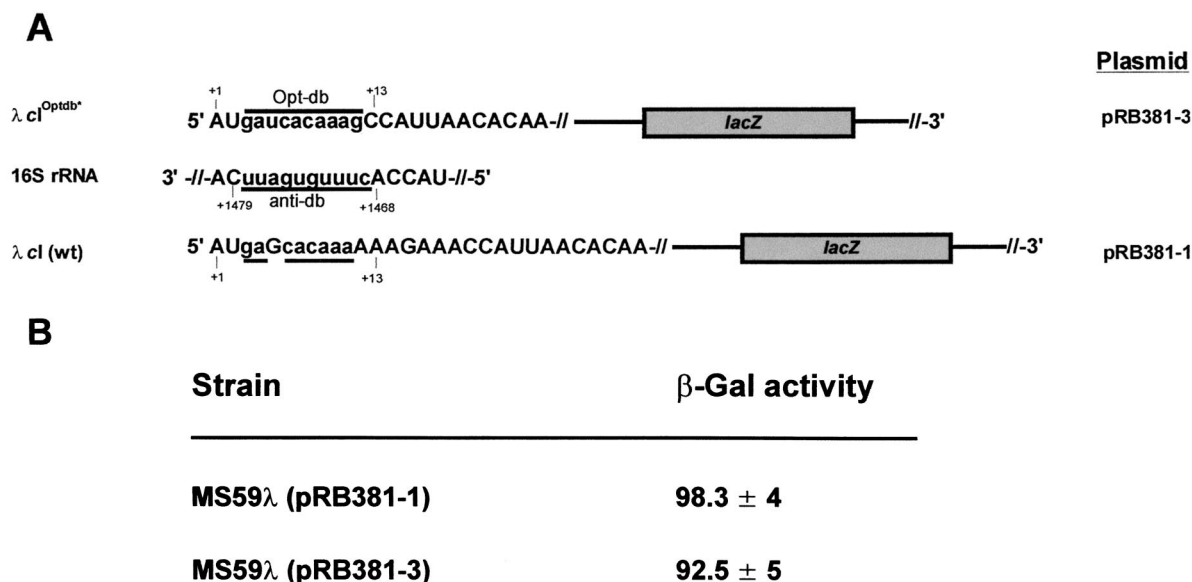


FIG. 3. Expression rates of the *cI-lacZ* and the *cI^{Optdb}-lacZ* constructs. (A) Possible db-*adb* complementarity in the *cI-lacZ* and the *cI^{Optdb}-lacZ* mRNA, respectively. (B) *E. coli* strain MS59 (λ wt) cells harboring plasmids pRB381-1 and pRB381-3, respectively, were grown in Luria-Bertani medium to an OD₆₀₀ of 0.8. Then, triplicate samples were taken from each culture and the β -galactosidase (given in Miller units) values were determined. The two Lys codons (subsequence, 5'... AAGAAA... 3') in λ cI wt mRNA following the putative db were omitted in the initial coding region of the *cI^{Optdb}-lacZ* construct.

1) should restrict a simultaneous interaction of both the start codon and anti-codon in the P site and the db-*adb*, it was not surprising that the presence of the optimal db did not enhance ternary complex formation (Fig. 2).

The failure of 30S ribosomes to form a binary complex with λ *cI^{Optdb}* 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 *cI* 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 putative *adb* (Fig. 3A). In both plasmids, the λ *P_{RM}* promoter abutted the start codon of the *cI* gene, which ensured that *cI* transcription started at the adenosine of the AUG initiating codon (see Materials and Methods). As shown in Fig. 3B, the optimized db did not increase translation of the *cI^{Optdb}-lacZ*

construct in vivo when compared to the corresponding *cI-lacZ* fusion, which corroborates the in vitro results presented in Fig. 2.

Expression of leaderless mRNAs in heterologous translation systems. Helix 44 is a phylogenetically conserved element in ribosomes. However, the rRNA sequence of the region corresponding to the putative *E. coli* *adb* is dissimilar in different bacteria and archaea. If the db has any significance in translation initiation, the diminished db-*adb* base pairing potential in a heterologous system would be expected to affect translation in a negative manner. Depending on the alignment, the initial coding region of λ *cI* mRNA shows, at best, a 4-nt complementarity with the *adb* of *B. subtilis* (Fig. 4A). Therefore, we asked whether a *cI-lacZ* reporter gene carried by plasmid pRB381cI (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 \pm 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

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