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Translation during cold adaptation does not involve mRNA–rRNA base pairing through the downstream box

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ABSTRACT

The downstream box (DB) has been proposed to enhance translation of several mRNAs and to be a key element controlling the expression of cold-shocked mRNAs. However, the proposal that the DB exerts its effects through a base pairing interaction with the complementary anti-downstream box (antiDB) sequence (nt 1469–1483) located in the penultimate stem (helix 44) of 16S rRNA remains controversial. The existence of this interaction during initiation of protein synthesis under cold-shock conditions has been investigated in the present work using an *Escherichia coli* strain whose ribosomes lack the potential to base pair with mRNA because of a 12 bp inversion of the antiDB sequence in helix 44. Our results show that this strain is capable of cold acclimation, withstands cold shock, and its ribosomes translate mRNAs that contain or lack DB sequences with similar efficiency, comparable to that of the wild type. The structure of helix 44 in 30S ribosomal subunits from cells grown at 37 °C and from cells subjected to cold shock was also analyzed by binding a ^{32}P-labeled oligonucleotide complementary to the antiDB region and by chemical probing with DMS and kethoxal. Both approaches clearly indicate that this region is in a double-stranded conformation and therefore not available for base pairing with mRNA.

Keywords: cold shock; RNA–RNA interaction; 16S rRNA structure; translational enhancers; translation at low temperature

INTRODUCTION

During initiation of protein synthesis in prokaryotes, the 30S ribosomal subunit is responsible for the recognition of the mRNA translation initiation region (TIR). This interaction is generally favored by base pairing between the pyrimidine-rich Shine–Dalgarno (SD) sequence, located upstream of the initiation codon in the majority of the mRNAs, and the anti-SD sequence located near the 3′ end of the 16S rRNA (for reviews, see Gualerzi & Pon, 1990, 1996; Gualerzi et al., 2000). This base pairing, which is neither necessary nor sufficient for translation, ensures efficient translation by providing a high local concentration of the initiation triplet near the ribosomal P site (Calogero et al., 1988). Since the introduction of computer programs capable of identifying and aligning homologous nucleic acid sequences, many elements (translational enhancers) found in the TIR of the bacterial mRNAs have been suggested to enable or to stimulate translation by the ribosome (for a review, see McCarthy & Gualerzi, 1990).

One of these elements is the downstream box (DB), located downstream of the initiation codon in some bacterial and phage mRNAs (e.g., genes 0.3 and 10 of bacteriophage T7, λ cI, and *Escherichia coli lysU*, *glnS*, *rpoH*, and several cold-induced *csp* genes), which has been proposed to act as a translational signal and enhancer (for a review, see Sprengart & Porter, 1997). The mechanism proposed for the stimulation of translation is a base pairing interaction with an antiDB sequence (nt 1469–1483) of 16S rRNA (Sprengart et al., 1996). This hypothesis is based on mutagenesis studies that show that increasing or decreasing the

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complementarity results in increased or decreased expression levels, respectively, but the experimental data in support of the DB-antiDB interaction are, at best, very controversial. In fact, even though the DB-antiDB interaction was claimed to be crucial for leaderless mRNA (e.g., λ cI mRNA transcribed from the *pM* promoter), Resch et al. (1996) have demonstrated that mutagenesis of the DB element of this mRNA had no effect on translation. More importantly, chemical probing of the cI mRNA within translation initiation complexes showed no protection of the bases comprising the putative DB element of this mRNA, thus nullifying the criticism (Etchegaray & Inouye, 1999a) that the mutagenesis may have introduced new DB sequences.

Additional work carried out with CAT mRNA showed that even after simultaneous removal of the 5′ untranslated region and of the two potential DB elements, the expression of chloramphenicol acetyltransferase under the control of a strong constitutive promoter allowed cells to survive in the presence of up to 20–30 μg/mL of chloramphenicol (Odjakova et al., 1998). Further doubt concerning the DB base pairing model has been generated by the recent evidence that ribosomes carrying a mutation in 16S rRNA that reversed part of helix 44, including the antiDB region, translate several DB-containing mRNAs in vivo with the same efficiency as wild-type ribosomes (O'Connor et al., 1999).

The base pairing mechanism is also inconsistent with all existing 16S rRNA models that predict that the antiDB sequence is within a stable helical region of the rRNA and, therefore, not able to engage in base pairing (at least not Watson & Crick) with the template (Gualerzi et al., 2000, and references therein).

Although DB base pairing seems to be ruled out under normal conditions (optimal temperature of growth, wild-type genetic background), the topographically difficult task of base pairing DB and antiDB could be overcome in particular situations such as the absence of a ribosomal component like protein S2 (Shean & Gottesman, 1992), during heat shock (Nagai et al., 1991; Morita et al., 1999), or during cold shock that induces the expression of RNA helicases (Mitta et al., 1997; Etchegaray & Inouye, 1999b).

It has been suggested that cold shock induces a modification of the translational apparatus that allows the preferential translation of cold-shocked mRNAs (Brandi et al., 1996; Goldenberg et al., 1997). The molecular nature of this modification has not yet been identified, but an interesting possibility is that cold-shocked ribosomes may be modified to allow a DB-antiDB base pairing interaction to occur, thereby permitting the selective translation of cold-shocked mRNAs. In fact, several publications from the same laboratory (e.g., Mitta et al., 1997; Etchegaray & Inouye, 1999b) have indicated that the DB base pairing interaction plays a critical role in the expression of cold-shocked proteins at low temperature. It has been reported, for instance, that the 14 nt DB element located 12 bases downstream of the initiation codon of the CspA mRNA is crucial, in concert with SD sequence, for the mRNA translation during cold shock. It has been claimed that in the absence of "cold-shocked ribosomal factors" it is the presence of the DB sequence that allows cold-shocked mRNAs to form initiation complexes at low temperature, whereas the non-cold-shocked mRNAs that lack this sequence remain translationally blocked. Furthermore, because it has been reported that one of the first proteins synthesized during cold shock is a ribosome-associated RNA helicase (Jones et al., 1996; Goldenberg et al., 1997), the possibility that cold-shocked ribosomes might expose the antiDB sequence, thereby allowing base pairing with the DB element present in cold-shocked mRNA, seemed to be worth testing.

In this article, we have tested the translation in vivo and in vitro of CspA mRNA, the accessibility of the antiDB sequence of 16S rRNA, and the cell survival after cold shock using both wild-type and the antiDB mutant *E. coli* strains and ribosomes (O'Connor et al., 1999). Our results demonstrate that DB-antiDB base pairing plays no role in CspA mRNA translation, is not relevant for cell survival after cold shock, and that the antiDB sequence of 16S rRNA is equally inaccessible in ribosomes from cold-shocked cells and control cells.

RESULTS

To test the hypothesis of a DB-antiDB interaction during translation initiation, the single copy of the *rrnB* operon carried by the *E. coli* strain constructed by Asai et al. (1999) has been mutagenized by O'Connor et al. (1999) to reverse the 12 bp helix containing the putative anti-downstream box (antiDB-flip mutation) thus creating a stem that has the same base composition and stability as the wild-type 16S, but which has altered the possibility to base pair with DB-containing mRNA (Fig. 1). Experiments carried out using this mutant strain showed that no DB base pairing takes place in vivo under normal growth conditions (O'Connor et al., 1999). However, as mentioned in the Introduction, the situation could be quite different during cold shock when the translation apparatus is modified to translate preferentially cold-shocked mRNAs (Brandi et al., 1996; Goldenberg et al., 1997) whose high translatability has been attributed to the presence of DB sequences (Mitta et al., 1997; Etchegaray & Inouye, 1999b).

In the following experiments, the ability of wild-type ribosomes to form initiation complexes and to translate mRNAs with and without DB sequences was compared to that of the ribosomes carrying the antiDB-flip mutation. Furthermore, to test the hypothesis that the antiDB sequence might become exposed only following ribosomal changes induced by cold shock,

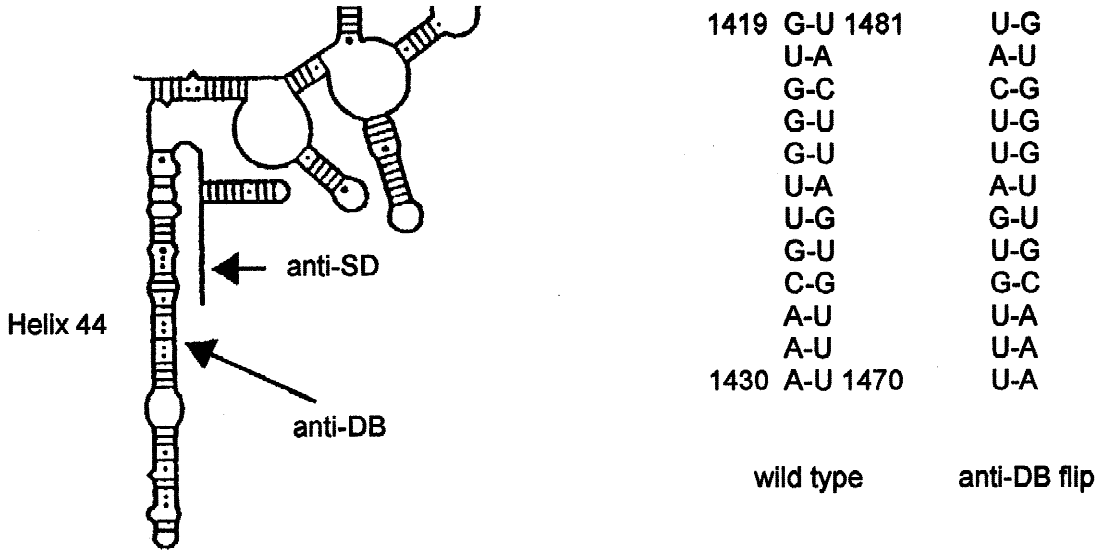

FIGURE 1. The 3′ end region of 16S rRNA comprising the last (45) and the penultimate (44) helices (left) and the sequences of wild-type and antiDB-flip rRNA mutants (right). Adapted from O'Connor et al. (1999).

we have also compared the activity of wild-type and mutant ribosomes prepared from control and cold-shocked cells.

Effect of the antiDB-flip mutation on 30S initiation complex formation

Binding of fMet-tRNA to the different types of 30S ribosomal subunits (wild-type and mutant, prepared from both control and cold-shocked cells) was compared in the experiment shown in Figure 2 and found to be essentially similar in all cases. In this experiment, the ribosomes were programmed either with a typical cold shock, DB-containing (10 bases of complementarity to the antiDB) mRNA like the CspA mRNA (Mitta et al., 1997) or with the mRNA encoding the nucleoid-associated protein HUα, which is regarded as a non-cold-shocked mRNA containing no significant DB sequence ($\leq$6 bases of complementarity within the first 25 nucleotides of the coding sequence). As seen in Figure 2, the wild-type control 30S ribosomes (Fig. 2A) bind almost the same amount of f[^{35}S]Met-tRNA in response to HUα mRNA and to CspA mRNA; qualitatively similar results are obtained with the subunits carrying the antiDB-flip mutation (Fig. 2B). The somewhat better activity displayed by cold-shocked ribosomes when programmed with a cold-shocked mRNA is in agreement with previous data (Brandi et al., 1996), whereas the slightly reduced ($<$30%) activity of the antiDB-flip mutants indicates that this mutation may have a generalized adverse effect on ribosomal function. A similar conclusion is also suggested by results presented below (Fig. 4).

Effect of the antiDB-flip mutation on in vitro translation of mRNAs with or without DB sequences

The experiments shown in the above section demonstrate that a potential DB-antiDB interaction does not influence the level of 30S initiation complex formed at equilibrium with saturating amounts of mRNAs. However, because the 30S initiation complexes represent a kinetic intermediate in the translation pathway (La Teana et al., 1993), it was also necessary to investigate whether a DB-antiDB interaction could affect translation, possibly by influencing the kinetics of the initiation phase of protein synthesis.

Thus, the in vitro translational activity of the wild-type and mutant ribosomes was tested using S30 extracts (Fig. 3A,B,C) or 70S ribosomes and S100 postribosomal supernatant (Fig. 3D) prepared from the wild-type and antiDB-flip mutant strains. Of the four mRNAs used to program these translation systems, two encode for typical cold-shocked proteins (CspA and H-NS) and contain a strong (CspA mRNA) and a weak (H-NS mRNA) DB sequence (Mitta et al., 1997), and the other two encode non-cold-shocked proteins (HUβ and IF1) and do not contain a significant DB sequence. As seen in Figure 3, although the S30 from wild-type, cold-shocked cells seems to be the most active translational system, overall, the translational activity of all types of S30 extracts and ribosomes does not significantly differ regardless of the mRNA used. Taken together, these results indicate that the flip mutation that would prevent base pairing between DB and anti-DB does not result in a selective loss of ribosomal activity vis-à-vis DB-containing mRNAs such as the CspA mRNA. The slightly

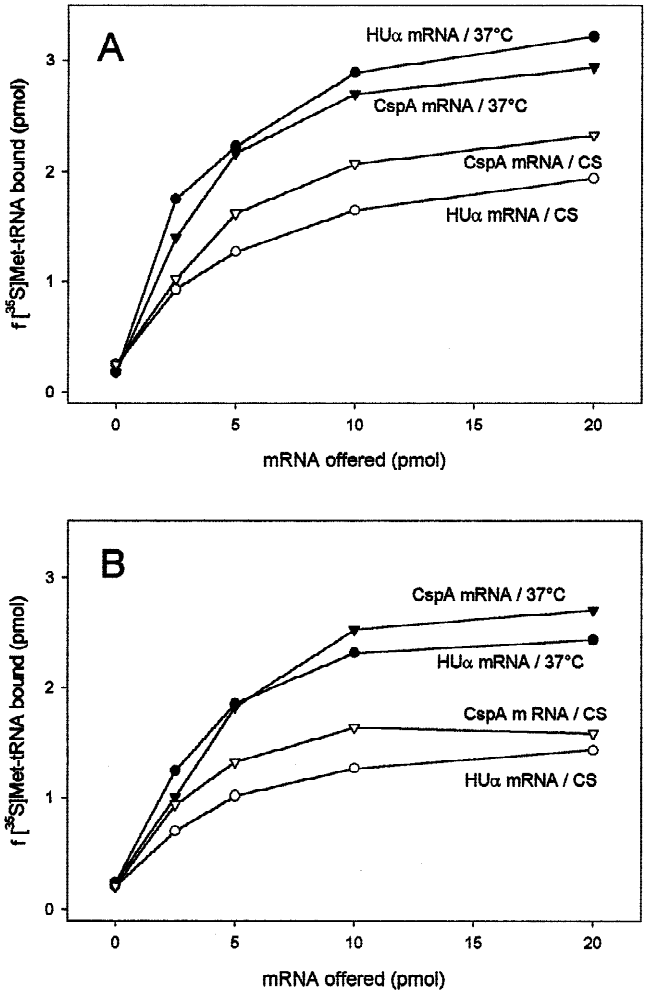

FIGURE 2. f[^{35}S]Met-tRNA binding to 30S ribosomal subunits. 30S initiation complexes were formed as described in Materials and Methods with CspA or HUα mRNA using wild-type (**A**) or antiDB-flip mutant (**B**) ribosomal subunits from cells grown at 37 °C (37 °C) or cold shocked (CS) as indicated in the figure.

reduced activity of these mutant ribosomes, like their reduced fMet-tRNA binding capacity (Fig. 2), can be explained by the introduction of a structural alteration that nonselectively affects the ribosomal function (see Fig. 4).

Accessibility of helix 44 and antiSD sequence of 16S rRNA in wild-type and antiDB-flip mutant 30S

To test the availability of the antiDB sequence in 16S rRNA for base pairing in control (Fig. 4A) and cold-shocked (Fig. 4B) 30S subunits (both wild type and mutant), we used an oligonucleotide complementary to the 1468–1483 region (DB probe). In these experiments we also used an oligonucleotide complementary to the anti-SD sequence (SD probe) to test the accessibility of this region of the 16S rRNA. Binding of the latter probe served as a control for the accessibility of the antiDB region, also in light of the claim that translation in the cold requires the DB-antiDB base pairing in combination with the SD-antiSD interaction (Mitta et al., 1997; Etchegaray & Inouye, 1999b). As shown in Figure 4, the antiDB sequence was totally inaccessible for base pairing with the DB probe not only in the mutant ribosomes, as expected, but also in the wild-type ribosomes prepared from both cold-shocked (Fig. 4B) and non-cold-shocked (Fig. 4A) cells. The same experiment demonstrates that the SD probe binds to both control and cold-shocked wild-type 30S subunits with the expected 1:1 stoichiometry, as evidenced by the maximum of 10 pmol of probe bound to 10 pmol of 30S subunits in the reaction mixture. Interestingly, the antiSD sequence is less accessible in the antiDB-flip mutant 30S prepared from both control (Fig. 4A) and cold-shocked cells (Fig. 4B). This result indicates that the helix 44 inversion is most likely responsible for a structural change of the subunit that is reflected in a less efficient SD-antiSD interaction. In turn, this finding may help to explain the somewhat reduced activity of the mutant compared to wild-type ribosomes observed in initiation complex formation (Fig. 2) and mRNA translation (Fig. 3). Reduced activity of the mutant ribosomes, entirely unrelated to a possible DB function, can also be expected on the basis of reports indicating that helix 44 is involved in subunit interaction (Firpo & Dahlberg, 1998; Merryman et al., 1999).

To ascertain whether the antiDB region could become accessible upon SD-antiSD interaction, additional experiments were performed in which the SD and DB probes were added together. The results obtained demonstrated that the presence of the SD probe had no effect on the accessibility of the antiDB region in either wild-type or mutant ribosomes (not shown).

Chemical probing of helix 44 in control and cold-shocked 30S ribosomal subunits

To confirm the inaccessibility of helix 44 seen in the previous section, the reactivity of this 16S rRNA helix towards dimethyl sulfate (DMS), to probe specifically A at N-1 and C at N-3 and kethoxal, to probe G at N-1 and N-2 was tested in 30S ribosomal subunits from control (Fig. 5, lanes 1) and cold-shocked (Fig. 5, lanes 2) cells. The primer extension analysis of helix 44 using an oligonucleotide complementary to the 1501–1539 region that allows the detection of modified nucleotides on both sides of the helix (positions 1417–1431 and 1469–1483) demonstrates, in agreement with that reported by Merryman et al. (1999), that no modification by the base-specific chemical probes occurs in the region encompassing the antiDB sequence in the control ribosomes. In addition, our results demonstrate that the same antiDB sequence is sequestered in a double-stranded structure and not accessible to the

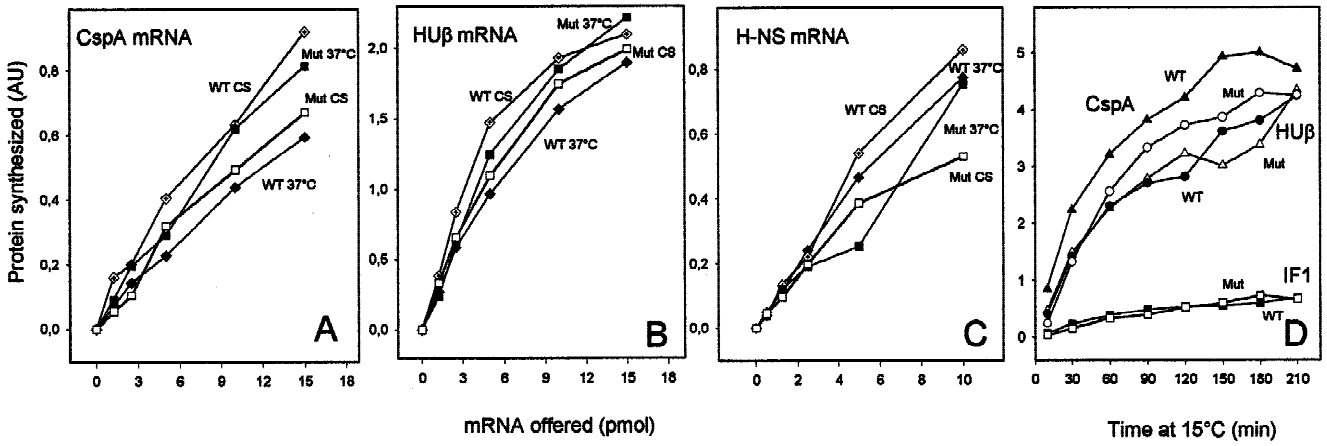

FIGURE 3. In vitro translation of CspA mRNA (**A**), HUβ mRNA (**B**) or H-NS mRNA (**C**) was performed as described in Materials and Methods using extracts (S30) obtained from cells with control (WT ♦,◇) or antiDB-flip (mut ■,□) 30S ribosomal subunits before (37 °C, filled symbols) and after cold shock (CS, open symbols). **D:** Time course of CspA (▲,△), HUβ (●,○), and IF1 (■,□) mRNA translation at 15 °C with wild-type (filled symbols) and antiDB-flip (open symbols) ribosomes. The experiment was carried out as described in Materials and Methods.

modifying agents in cold-shocked ribosomes, indicating that this stress does not cause unwinding of helix 44.

Effect of the antiDB-flip mutation on cold adaptation in vivo

Taken together, the in vitro experiments shown above demonstrate that a base pairing interaction between the DB sequence of the mRNA and the potentially complementary sequence in the 16S rRNA (antiDB sequence) does not and cannot occur. The experiments shown in this and in the following section are aimed at determining whether similar conclusions could also apply in vivo under cold-shock conditions. Thus, we have determined the effect of the antiDB-flip mutation on cell growth at low temperature and cell survival after cold shock. For this purpose, both wild-type and mutant strains, grown at 37 °C in LB to an $A_{620} \cong 0.5$, were shifted to 15 °C, after which the change in optical density of the two cultures at 620 nm was followed (Fig. 6A)

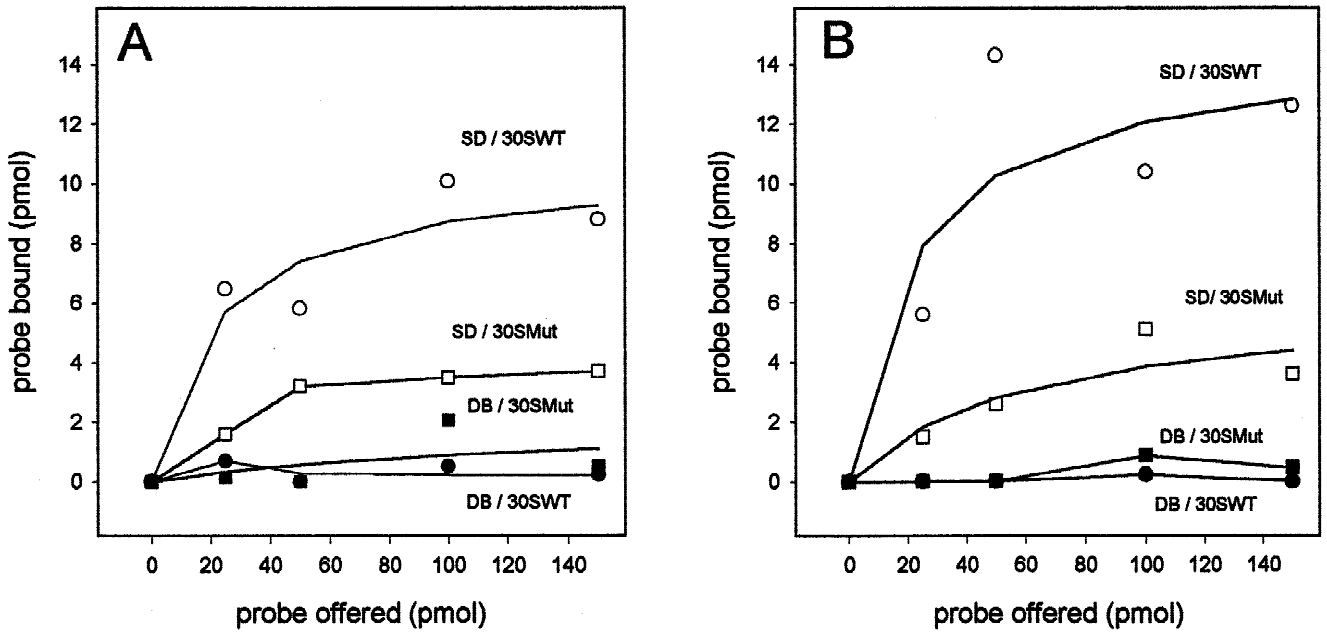

FIGURE 4. Accessibility of 30S subunits to antiSD and antiDB probes. The ^{32}P-labeled probes were bound to wild-type (WT) or anti DB-flip mutant (Mut) 30S ribosomal subunits from cells grown at 37 °C (**A**) or from cells subjected to cold shock (**B**). The experimental procedure is described in Materials and Methods.

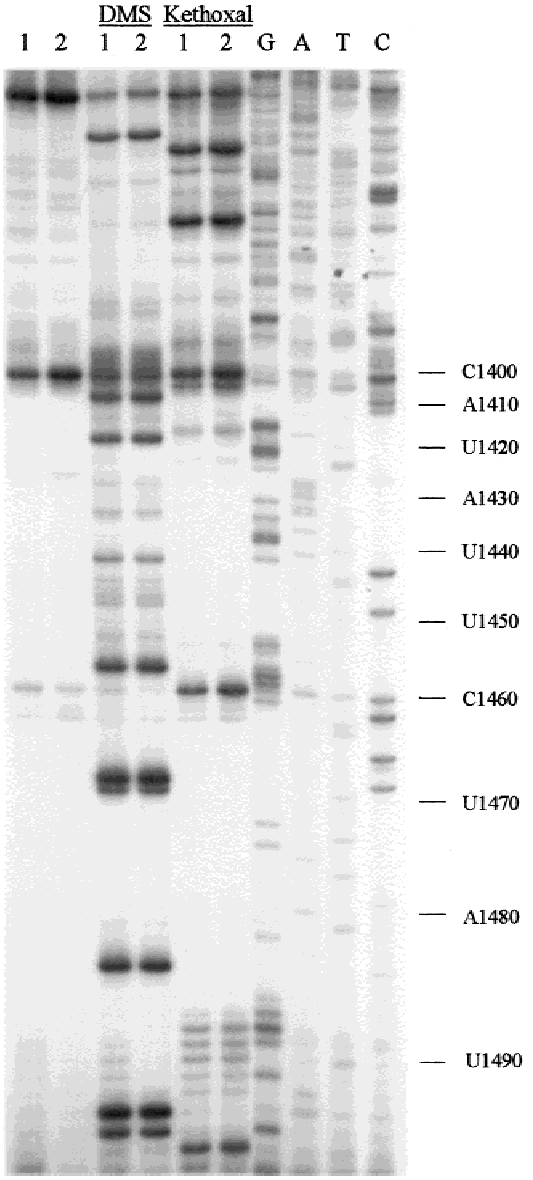

FIGURE 5. Chemical probing of helix 44 in wild-type 30S ribosomal subunits from cells grown at 37 °C (lanes 1) or from cells subjected to cold shock (lanes 2). The probing and primer extension reactions were performed as described in Materials and Methods. The first two lanes 1 and 2 are primer extension reactions of unmodified rRNA. Lanes G,A,T,C are the sequencing lanes using 16S rRNA as the template.

and the number of viable cells (Fig. 6B) determined. As seen from Figure 6, the growth rate of the mutant strain (doubling time ≅ 26.4 h) is slightly slower than that (≅21.2 h) of the corresponding wild-type strain; this represents a 24% decrease, only slightly more than the 15% decrease observed at 37 °C under normal growth conditions (O'Connor et al., 1999). After roughly three generations (60–70 h) both cultures reach the same optical density. The cell viability measured in samples taken at different times following cold shock indicates that the antiDB-flip mutation causes the cells to become somewhat more vulnerable; after a delay corresponding roughly to a single generation, however, the mutation does not prevent the cells from growing and multiplying in the cold to a level that is ultimately similar to that reached by wild-type cells. This delay seems to be caused mainly by the initial drop in cell viability, which is more drastic in mutant than in wild-type cells.

Expression of a cspA:β-galactosidase fusion during cold shock

Strain TA548 containing pMO10wt or pMO10mDB (O'Connor et al., 1999) was transformed with a *cspA-lacZ* fusion constructed in plasmid pMC1871. Each strain was then incubated at 37 °C to an $A_{600} = 1.0$ and then shifted to 15 °C; the levels of β-galactosidase activity expressed by these cells, monitored at different times after the temperature shift, are presented in Figure 7. The results obtained clearly indicate that the antiDB-flip mutation does not inhibit the in vivo translation of the mRNA encoding the fusion protein that begins with the cold-shock, DB-containing *cspA* sequence.

DISCUSSION

The DB was originally proposed to act synergistically with the SD sequence to ensure efficient translation of some mRNAs or, when alone, to compensate for the lack of an SD sequence under otherwise normal conditions of translation (Sprengart et al., 1996). The evidence purporting the participation of the DB element in translation invariably entails (both in the premises and in the interpretation of the experimental results) the formation of Watson–Crick base pairs between the DB and the antiDB sequence (nucleotides 1469–1483) located in the penultimate helix (helix 44) of 16S rRNA.

Recently O'Connor et al. (1999) have challenged this hypothesis by showing that an *E. coli* strain expressing only the antiDB flip rRNA can sustain the expression of several DB-containing mRNAs to the same extent as the wild-type strain, in spite of its inability to base pair with the DB element of these mRNAs. As mentioned in the introduction, these results do not rule out the possibility that DB-antiDB pairing might occur in ribosomes obtained from cold-shocked cells which contain cold-induced RNA helicases and RNA chaperones.

To analyze the actual feasibility of a DB-antiDB interaction with cold-shocked ribosomes, in this study we have used the mutant strain constructed by O'Connor et al. (1999) to ascertain whether such base pairing could indeed occur during cold adaptation. Using the very same type of approach that has been successfully used in the past to demonstrate the occurrence of the SD-antiSD base pairing in vivo, we demonstrate that no DB-antiDB interaction occurs, either in vitro or in vivo. In fact, our results in vitro clearly indicate that wild-type and antiDB-flip mutant ribosomes from both control and cold-shocked cells display similar activities

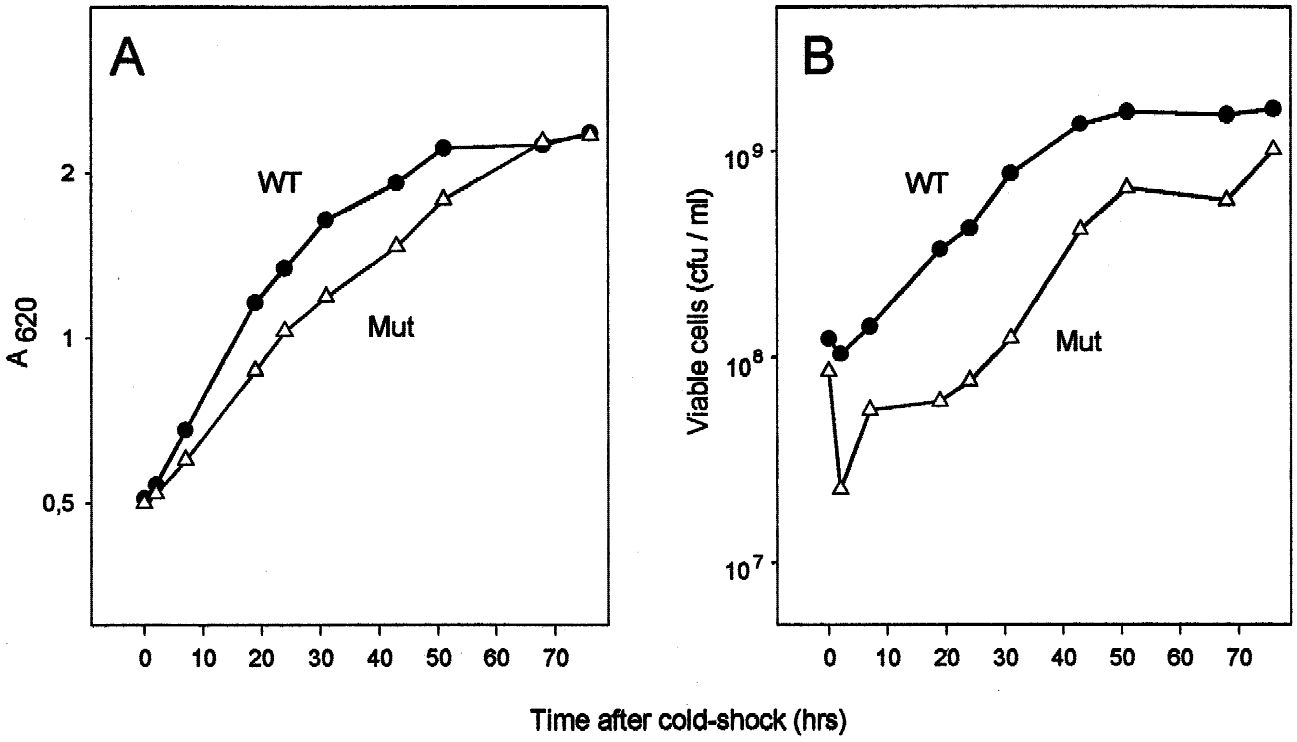

FIGURE 6. Growth curve (**A**) and cell viability (**B**) of the antiDB-flip mutant strain and of its isogenic wild-type strain under cold-shock conditions. The two cultures were grown at 37 °C to A_{620} = 0.5 and then shifted to 15 °C for the indicated times. The number of viable cells was determined as described in Materials and Methods. The experiment shown in **A** was repeated three times and that in **B** two times with almost identical results.

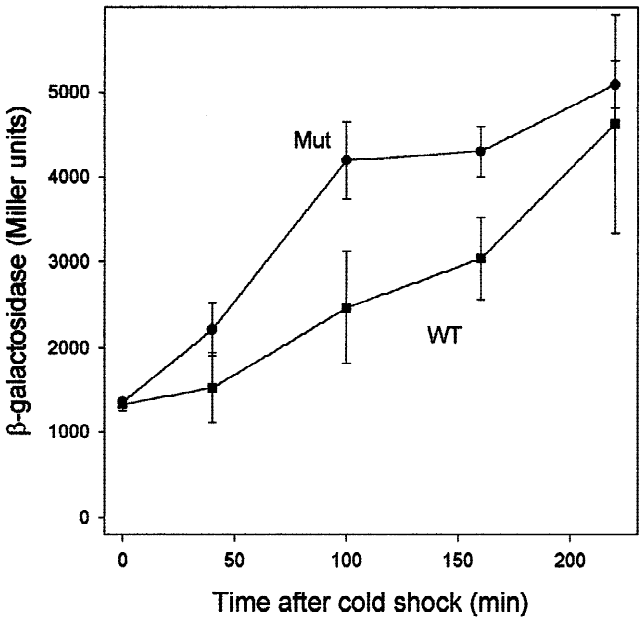

FIGURE 7. *β*-Galactosidase expression from a *cspA:β-gal* fusion during cold shock in cells containing control (WT) or antiDB-flip (Mut) 30S subunits. The experiment was performed as described in Materials and Methods. Each point represents the average of samples taken from four different cultures of each strain.

in initiation complex formation as well as in protein synthesis directed by cold-shocked and non-cold-shocked mRNAs, with or without extended DB sequences. In addition, in vivo, the mutant ribosomes were found to translate a reporter protein expressed from an mRNA construct containing an extended DB sequence with at least the same efficiency as wild-type ribosomes, and the cells harboring exclusively the mutant ribosomes were shown to survive the cold stress.

The delay of the cells harboring the mutant ribosomes in growing out of cold shock and the somewhat reduced activity of these ribosomes in some experiments probably stem from their defect in associating with the 50S subunit (Firpo & Dahlberg, 1998) and from the reduced accessibility of their antiSD sequence (Fig. 4). In any case the activity of these ribosomes does not seem to correlate in any rational way with the potential occurrence of DB-antiDB interaction.

Consistent with these conclusions, the antiDB region of all ribosomes tested was shown to be inaccessible to a complementary oligonucleotide probe and similarly unreactive to chemical reagents like DMS or kethoxal. Taken together these results are fully compatible with all current structural models of the 30S subunit,

which place helix 44 far from the mRNA decoding region. In fact, according to Mueller et al. (1997), helix 44 is located on the interface side of the subunit, whereas the downstream end of the mRNA passes through the hole leading out to the solvent side so that the whole shoulder of the 30S is lying between the DB element of the mRNA and the antiDB sequence of helix 44, thus physically preventing contact between them. This premise is fully confirmed by the recently elucidated crystallographic structure of the 30S subunit that shows helix 44 running along the longitudinal axis of the body far away from the mRNA channel (A. Yonath & F. Franceschi, pers. comm.).

Finally, it should be mentioned that Rocha et al. (1999) have recently analyzed the coding sequences (from +4 to +33) of all *Bacillus subtilis* genes and of *E. coli* tRNA synthetase genes (the genes used for the definition of the downstream box) and failed to identify any statistically significant motif. Furthermore, upon randomizing the sequence of this region and checking for the maximal score that would be obtained by chance alone, these authors found that 34% of the random sequences had a DB with eight matches and 9% had one with nine matches.

In conclusion, a base pairing interaction involving the so-called DB sequence is highly improbable, if not physically impossible, not only under normal conditions of growth, but also during cold adaptation and the presence of DB elements in the mRNAs is not statistically significant.

MATERIALS AND METHODS

Preparations

The S30 fractions, S100 and 70S ribosomes were prepared as described (Brandi et al., 1996) from *E. coli* mutant strain TA531 (Δ*rrnE* Δ*rrnB* Δ*rrnA* Δ*rrnH* Δ*rrnG::lacZ* Δ*rrnC::cat* Δ*rrnD::cat* Δ*recA* /pTRNA66 pHKrrnC), carrying deletions in all seven chromosomal *rrn* operons and expressing total wild-type or mutant rRNA from plasmids pKK3535 wild-type or from its antiDB-flip mutant derivative, respectively (O'Connor et al., 1999). Ribosomal subunits and fMet-tRNA were prepared as previously described (Ohsawa & Gualerzi, 1983). Messenger RNAs were prepared by in vitro transcription (Brandi et al., 1996) of the corresponding genes cloned in pTZ18R under the control of the T7 promoter.

S30 extracts were obtained from the wild-type and mutant strains grown at 37 °C in LB supplemented with ampicillin (50 μg/mL). At an A_{620} = 1.0, half of each culture was harvested, and the rest was incubated at 10 °C for 90 min.

In vitro translation was performed essentially as described in Brandi et al. (1996). Reactions containing S30 were carried out in 25 μL using an amount of S30 corresponding to about 6 pmol of ribosomes with the indicated templates; after 180 min at 15 °C, 10 μL from each reaction mixture were spotted onto Whatman 3MM discs that were processed in hot trichloroacetic acid. The in vitro protein synthesis was followed as the incorporation of [^{14}C]lysine.

The time-course experiment was carried out at 15 °C using 70S ribosomes and S100 extracts from cells grown at 37 °C to a cell density of 0.7 (A_{620}) and then incubated at 15 °C for 60 min. Each reaction mixture contained, in 100 μL, 60 pmol of ribosomes, 20 pmol of mRNA and [^{35}S]methionine as the radioactive amino acid. At the indicated times, 20 μL from each reaction mixture were withdrawn and loaded onto a 18% PAGE SDS-gel. The radioactive products were analyzed using a BioRad GS-250 Molecular Imager.

Binding of fMet-tRNA to 30S ribosomal subunits

Binding of f[^{35}S]Met-tRNA to 30S ribosomal subunits was performed essentially as described (Ohsawa & Gualerzi, 1983). Ten picomoles of each type of 30S subunits were incubated in the presence of 10 pmol of f[^{35}S]Met-tRNA and increasing amount of the indicated mRNAs. The reactions containing 30S from cells grown at 37 °C were incubated at 37 °C for 15 min; reactions containing cold-shocked ribosomes were incubated at 15 °C for 45 min.

Oligonucleotide binding to 30S ribosomal subunits

The antiSD probe (5′-AAGGAGGT-3′) and antiDB probe (5′-TCATGAATCACAAAG-3′) were 5′ labeled using γ-^{32}P-ATP and T4 kinase and incubated in the presence of wild-type or antiDB-flip mutant 30S ribosomal subunits. Reactions were carried out in 50 μL of 20 mM Tris-HCl, pH 7.7 buffer containing 15 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, 10 pmol of 30S subunits and increasing amounts of each oligonucleotide. After incubation at 15 °C for 45 min, the extent of binding was measured by filtration on Millipore discs, and filters were washed with 3 mL of the same reaction buffer, dried, and counted. Control reactions were run in the absence of 30S ribosomal subunits and background radioactivity was subtracted.

Chemical probing of 16S rRNA in 30S ribosomal subunits

Probing of the 16S rRNA was performed with DMS (Kodak) and kethoxal (Research Organics) essentially as described (Moazed & Noller, 1986) with the exception that the modification reaction of the cold-shocked 30S subunits was carried out at 15 °C for 30 min.

An oligonucleotide complementary to region 1501–1539 of 16S rRNA was used as a primer for the reverse transcriptase reaction.

Determination of cell growth and viability

Cultures of the *E. coli* mutant strain TA531 expressing wild-type or antiDB mutant rRNA (O'Connor et al., 1999) were grown at 37 °C in LB medium supplemented with 50 μg/mL ampicillin. At an A_{620} = 0.5, the cultures were shifted to 15 °C. Cell viability of the two strains, before and after the temperature shift, was determined from aliquots withdrawn at different times and plated on LB. Each point was performed in

duplicate. The number of cells generating colonies was calculated after incubation at 37 °C.

Construction and analysis of a *cspA-lacZ* fusion

A 386-bp fragment of the *cspA* gene was amplified from chromosomal DNA using the primers: CspA frwd (GCGTTTGAT TCAAGCCAACCCGGGATTAAGTAAGCAG) and *cspA* reverse (GCCGAAGCCTTTCCCGGGGTTGAACCATTTTAC GATACC). The forward primer annealed 330 bases upstream of the *cspA* initiation codon and the reverse primer annealed to a region 54 bases downstream of the initiation codon. Each of these primers incorporated a recognition site for *Xma*I to permit cloning of the amplified fragment into the fusion vector pMC1871 (Shapira et al., 1983). The amplified fragment includes both the major *cspA* promoters and the region downstream of the AUG codon shown to be important for *cspA* translation (Mitta et al., 1997). The amplified fragment was purified on Qiagen columns, digested with *Xma*I and ligated to *Xma*I-cleaved pMC1871. MC1061 cells were transformed with this ligation mixture, the transformants were plated on medium containing tetracycline and X-gal and several blue, tetracycline resistant transformants were analyzed by DNA sequencing. The fusion plasmids carrying the correct *cspA* sequences were designated pcspAZ.

Derivatives of strain TA548 (ΔrrnE ΔrrnB ΔrrnA ΔrrnH ΔrrnG::cat ΔrrnC::cat ΔrrnD::cat recA56 /pTRNA66, pMO10 wt/pMO10mDB) carrying deletions in all seven chromosomal *rrn* operons and expressing total wild-type or antiDB flip mutant rRNA from plasmids pMO10wt and pMO10mDB, respectively (O'Connor et al., 1999), were transformed with pcspAZ. These strains were grown in minimal glucose medium supplemented with casamino acids, thiamine, adenine, methionine, neomycin, and tetracycline at 37 °C until an OD_{600} of 1.0 was reached. The cultures were then shifted to 15 °C and β-galactosidase activity was measured (Miller, 1972) thereafter at hourly intervals.

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