Translational Enhancement by an Element Downstream of the Initiation Codon in *Escherichia coli*  

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The translation initiation of *Escherichia coli* mRNAs is known to be facilitated by a cis element upstream of the initiation codon, called the Shine-Dalgarno (SD) sequence. This region of the RNA sequence designated as the downstream box (DB) is complementary to bases 1469–1483 within the *Escherichia coli* 16 S rRNA (anti-DB sequence). It is speculated that formation of a duplex between the DB and anti-DB is responsible for translational enhancement (2). The DB sequence has also been implicated in the translation of the λc1 mRNA, an mRNA that lacks any untranslated region and the SD sequence (3). Interestingly, λc1 translation was enhanced at 42 °C in a temperature-sensitive strain in which the amount of ribosomal protein S2 decreased at 42 °C. It was proposed that the anti-DB sequence in S2-deficient ribosomes indirectly becomes more accessible to DB, resulting in enhancement of translation initiation of the λc1 mRNA (3, 4). However, the role of the DB in λc1 translation initiation was disputed by Resch and co-workers (5). These authors constructed lacZ translational fusions with the λc1 gene to test the DB function. Since a deletion of 6 bases encompassing a portion of the DB sequence did not reduce the formation of the translation initiation complex, they disputed the existence of DB. Despite these elusive roles of DB (6), we have shown that the presence of a DB sequence in cold-shock mRNAs plays an important role in translation efficiency, and we proposed that the DB is involved in the formation of a stable initiation complex at low temperature before the induction of cold ribosomal factors (7). Furthermore, we pointed out the SELEX enrichment of DB-like sequences in an mRNA when the 30 S ribosomal subunit was used as a ligand (8, 9).

It was suggested that the results obtained by Resch et al. (5) could be explained by recreating a new DB as a result of the deletion of the original DB in the λc1 mRNA (6). Indeed, a 6-base deletion eliminating 5 out of 8 matches in the original DB recreated a new 9-base matching DB sequence including the initiation codon. Therefore, the results by Resch and collaborators (5) could be explained by the newly created DB. In the present paper, we performed both biochemical and genetic experiments to reexamine the role of the DB in translation initiation, and we determined that DB plays a crucial role in regulation of gene expression in *E. coli* by enhancing the formation of the translation initiation complex.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction—**The expA-lacZ fusion constructs were made by the insertion of annealed oligonucleotides at the EcoRI site of the pJJG78 (10). Annealed oligonucleotides DB1 (5′-AATTAAATCACAAG-TGGG-3′) with DB1 (5′-AATCCCAACTTTGATTT-3′) or DB2 (5′-AAAATATGATCAAAAGTGGG-3′) with DB2 (5′-AATCCCAACTTTGATTT-3′) were used to create pJJG78DB1 and pJJG78DB2 constructs, respectively.

The pIN-lacZ constructs were made by inserting the *XbaI* fragment from pJJG78 or pJJG78DB2 into the *XbaI* site of pIN-III (10) to create pINZ and pINZDB1, respectively. Then, the annealed oligonucleotides ZDB2 (5′-CTAGCCCTTTAATTTGAATCACAAAGTGGG-3′) with ZDB2 (5′-AATCCCAACTTTGATTT-3′) were used to create pINZDB2. Annealed oligonucleotides ZDB3 (5′-CTAGCCCTTTAATTTGAATCACAAAGTGGG-3′) with ZDB3 (5′-AATCCCAACTTTGATTT-3′) or ZDB4 (5′-CTAGCCCTTTAATTTGAATCACAAAGTGGG-3′) with ZDB4 (5′-AATCCCAACTTTGATTT-3′) were inserted at the *XbaI* site of pINZ to construct pINZDB3 and pINZDB4, respectively.

**β-Galactosidase Activity—** *E. coli* AR137 (pconcB′) (11) or JM38 (pconcB′) harboring different plasmids were grown at 37 °C to mid-log phase in 20 ml of LB medium containing 50 μg/ml ampicillin in a 125-ml flask. The cultures were then transferred to a 15 °C shaking water bath, or isopropyl-β-d-thiogalactopyranoside (IPTG, 1 mg/ml) was added to a final concentration of 1 mg/ml. A 100-μl culture was taken at each time point. β-Galactosidase activity was measured according to Miller’s procedure (12).

**Prime Extension—** *E. coli* AR137 (pconcB′) or JM38 (pconcB′) carrying different plasmids were grown under the same condition as used for the β-galactosidase assay described above. To estimate the mRNA amounts of the *lacZ* fusion constructs, 1.5 ml of culture was taken at each time point, and total RNA was extracted by the hot phenol method (13).

For the mRNA stability experiments at 15 °C, rifampicin (0.2 mg/ml) was added 30 min after the temperature downshift from 37 °C. The mRNA amounts from the expA-lacZ fusion constructs were estimated by primer extension using the 32P-labeled M13-47 antisense primer complementary to the region of *lacZ* between codons 14 and 22 as described previously (7).

The reverse transcription reaction was carried out with AMV-RT according to the manufacturer’s procedure (Boehringer Mannheim), and the cDNA products were resolved on a 6% Sequencing Gel and quantified by PhosphorImager (Bio-Rad).

**Pulse Labeling—** Cultures of *E. coli* AR137 (pconcB′) cells carrying pINZ or pINZDB1 were grown at 37 °C under the same conditions used after the pulse-labeling.
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**RESULTS**

The Effect of a Perfectly Matching DB—It has been previously shown that DB is essential for the production of CspA at low temperature (7). However, the wild-type DB of cspA has 10 matches out of 15 possible matches to the anti-DB in 16 S rRNA (7). Therefore, we added DBs of 12 (pJJG78DB1) or 15 (pJJG78DB2) bases that are complementary with the anti-DB of 16 S rRNA to the site after the 5th codon of lacZ under the cspA regulatory system in pJJG78 (see Fig. 1A) to examine if they enhance lacZ expression at 15 °C. Mid-log phase cells (penB+) grown at 37 °C were shifted to 15 °C, and β-galactosidase activity was measured at 1, 2, and 3 h after the shift.

Fig. 1B shows that at 1 h at 15 °C the β-galactosidase activity was 3- and 8-fold higher with pJJG78DB1 and pJJG78DB2, respectively, than with pJJG78. After 2 and 3 h at 15 °C, the β-galactosidase activity was increased 3.5 and 10.5 times with pJJG78DB1 and pJJG78DB2, respectively, than with pJJG78 (Fig. 1B). Moreover, the effect of the DB was observed at 37 °C in which the β-galactosidase activity of pJJG78DB1 and pJJG78DB2 was 2- and 4-fold higher as compared with pJJG78. The amount of the lacZ mRNA (Fig. 1C) at each time point as well as the mRNA stability (Fig. 1D) did not vary significantly between these constructs. The lacZ mRNA half-life from pJJG78, pJJG78DB1, and pJJG78DB2 was calculated to be 27, 23, and 25 min, respectively. In addition, computer analysis (18) revealed no significant differences in the mRNA secondary structures among pJJG78, pJJG78DB1, and pJJG78DB2, suggesting that the insertion of the perfectly matching DB may not have a particular effect in the mRNA secondary structures that could account for the difference in their β-galactosidase expression. These results indicate that DB functions as a translational enhancer and that greater
complementarity to the anti-DB improves translational efficiency and/or that specific base pairings like the first three nucleotides of the DB from pJJG78DB2 may play an important role for the DB activity.

DB Functions at 37 °C—The experiments described above were carried out at 15 °C. In order to examine whether DB also works at 37 °C, the cspa cold-shock regulatory regions upstream of SD of pJJG78 and pJJG78DB2 were replaced with the constitutive lpp promoter and the lac promoter-operator fragment using a pINIII vector (19), yielding pINZ and pINZDB1, respectively (Fig. 2A). Cells (penB') transformed with pINZ or pINZDB1 showed very low β-galactosidase activity in the absence of IPTG, an inducer of the lac promoter (Fig. 2B, time 0). Upon the addition of 1 mM IPTG, β-galactosidase activity was induced in both cells. After 3 h induction, β-galactosidase activity increased 18- and 37-fold for pINZ and pINZDB1, respectively (Fig. 2A). Cells (penB') transformed with pINZ or pINZDB2 showed very low β-galactosidase activity in the absence of IPTG, an inducer of the lac promoter (Fig. 2B, time 0). Upon the addition of 1 mM IPTG, β-galactosidase activity was induced in both cells. After 3 h induction, β-galactosidase activity increased 18- and 37-fold for pINZ and pINZDB1, respectively (Fig. 2A). However, the levels of β-galactosidase activity show a dramatic difference between the two; the activity of pINZDB1 (34) was 34 times higher than that without DB (pINZ), demonstrating that DB functions at 37 °C as well. Specific activities of β-galactosidase produced from vector pINZ and pINZDB1 are almost identical (data not shown), and thus the addition of the five amino acid residues in the β-galactosidase sequence of pINZDB2 may play an important role for the DB activity. Furthermore, the stabilities of SD was removed by deletion of the 15-base sequence (codons 1–5) from pINZDB2 (Fig. 2A), β-galactosidase activity at all time points was reduced to the background level (Fig. 2B), indicating that the secondary SD played a crucial role in the translation of the pINZDB2 lacZ mRNA. When the SD sequence was recreated by 5-base substitution in pINZDB3 (Fig. 2C), β-galactosidase activity of this construct was recovered to a comparable level to that of pINZDB1 (Fig. 2B). This is important to notice that the DB sequence starting from the first AUG codon was eliminated in pINZDB4 (Fig. 2C). Therefore, the high expression of β-galactosidase from pINZDB1 and pINZDB4 is due to the perfectly matching DB sequence (Fig. 2B). These results indicate that (a) DB functions only in the presence of SD, and (b) the position of DB is flexible starting from either codon 1 or 6.

Enhancement of Protein Synthesis by DB—The β-galactosidase activity shown in Fig. 2B indicates that DB enhances the translation of pINZDB1. Therefore, in order to test the effect of the DB in translation efficiency, the rate of β-galactosidase synthesis from pINZ and pINZDB1 was analyzed. The rate of β-galactosidase synthesis was measured by pulse labeling cells for 5 min with [35S]methionine after the addition of IPTG using cells harboring pINZ and pINZDB1. After SDS-PAGE, the amounts of radioactive β-galactosidase were estimated using a PhosphorImager (Fig. 3). Prior to the addition of IPTG, the rate of β-galactosidase synthesis from pINZ and pINZDB1 was
Identical. However, upon IPTG induction the rates of β-galactosidase synthesis from pINZDB1 were continuously increasing at each time point, whereas the rate of β-galactosidase synthesis from pINZ was almost not affected. After 4 h of IPTG addition the rate of β-galactosidase synthesis from pINZDB1 was 6.5 times higher than that of pINZ. This result demonstrates that DB enhances the translation efficiency of pINZDB1 as reflected by the increment in the synthesis of β-galactosidase.

Enhancement of Translation Initiation by DB—In order to examine whether DB enhances translation initiation, we next analyze the ability of lacZ mRNA from pINZ and pINZDB1 to form polysomes. For this experiment, pcnB cells were used to amplify the effect of DB. Interestingly, cells with pINZDB1 could not form colonies on LB plates in the presence of 1 mM IPTG, whereas cells with pINZ formed colonies. The lethal effect of IPTG on the cells with pINZDB1 is considered to be due to overexpression of β-galactosidase. After the addition of IPTG, cell growth was stopped by the addition of chloramphenicol (0.1 mg/ml) at 15, 30, and 60 min, and then polysome profiles were examined as shown in Fig. 4. From each gradient fraction (500 µl), 200 µl were spotted on a nitrocellulose membrane, and the amount of the lacZ mRNA analyzed using a 24-base antisense oligonucleotide (M13–47 oligonucleotide). The amounts of the lacZ mRNA were quantified by a PhosphorImager and are displayed in Fig. 4. Although the polysome profiles are similar, there are significant differences in the distribution of the lacZ mRNA; at 15 min the lacZ mRNA mainly exists in the upper half of the gradient (fraction 8–14, corresponding to 70 S to 30 S ribosomes) with pINZ, while with pINZDB1 a major peak (fraction 3 to 8) is formed in the lower half of the gradient. At 30 min, the lacZ mRNA from pINZ moved to the position of the 70 S ribosome, whereas the lacZ mRNA from pINZDB1 maintained a similar pattern as that at 15 min. At 60 min a major fraction of the lacZ mRNA from pINZ remained in the upper half of the gradient, whereas the lacZ mRNA from pINZDB1 was broadly distributed from higher order polysomes to 70 S ribosome fraction. Therefore, the reason why cells harboring pINZDB1 could not form colonies on LB plates containing 1 mM IPTG may be due to a decrease in the concentration of free ribosomes as a result of the massive expression of a highly translatable DB-containing mRNA. These results indicate that DB enhances the efficiency of polysome formation probably due to a translation initiation enhancement.

In order to estimate the exact effect of DB from the above experiment, the amount of the lacZ mRNA and the β-galactosidase activity were measured at the same time points taken in the polysome profiles (15, 30, and 60 min after IPTG induction). As shown in Fig. 5A, the amounts of the lacZ mRNA reached almost the maximal level at 15 min for both pINZ and pINZDB1. The PhosphorImager analysis of this result revealed that the amounts of the pINZDB1 mRNA are 1.5, 1.4, and 1.3 times higher than those of the pINZ mRNA at 15, 30, and 60 min, respectively. The higher mRNA levels for pINZDB1 are probably attributable to the highly efficient polysome formation of pINZDB1 that may stabilize the mRNA (21). The induction of β-galactosidase activity is shown in Fig. 5B. In the case of pINZDB1, the activity is very high even in the absence of IPTG, and upon the addition of IPTG, it increased from 18,500 to 64,400 units (3.5-fold) after 2.5 h incubation. In the case of pINZ, the background activity prior to IPTG induction was much lower, and it increased from 500 to 2,900 units (3-fold) at the 2.5-h time point. The increment of the β-galactosidase activity of pINZDB1 between 30 and 60 min is 35 times higher than that of pINZ, and therefore the efficiency of β-galactosidase production for pINZDB1 is calculated to be 26 times higher than that for pINZ on the basis of the amount of mRNA. Therefore, the higher levels of β-galactosidase production from pINZDB1 are due to a high efficiency of polysome formation.

Next, in order to demonstrate more directly the translation-enhancement effect of DB, the β-galactosidase synthesis was examined in a cell-free system using pINZ and pINZDB1. The [35S]methionine incorporation into β-galactosidase (band G) with pINZDB1 (2nd lane, Fig. 6A) was 8-fold higher than that with pINZ (1st lane), whereas the β-lactamase (band L) production was almost identical in both lanes. Fig. 6B shows a time course of in vitro production of β-galactosidase from pINZ and pINZDB1 performed as described above. The same reaction was carried out with non-radioactive methionine, spotted on a nitrocellulose membrane, and hybridized with the M13–47 oligonucleotide as described under “Experimental Procedures.” As shown in Fig. 6C, at each time point the amount of lacZ mRNA from pINZ and pINZDB1 was almost identical. This result supports the role of the DB as a translational enhancer from the in vivo data described above.

Further Enhancement of DB-dependent Translation by S2°—It has been proposed that in the absence of ribosomal protein S2, structural changes in 16S rRNA result in the release of the anti-DB sequence from the penultimate stem making it more accessible to base pair with DB (3, 4). We analyzed the β-galactosidase expression of pINZ, and pINZDB1 in E. coli CS239 that carries an S2 temperature-sensitive mutation (3). Fig. 7A shows that the β-galactosidase activity of pINZDB1 significantly increases upon shifting the temperature from 30 to 42 °C in the S2° strain (CS239) (6.3-fold from 0 to 3.5 h), whereas the activity in the wild-type strain (CS240) slightly increased (1.1-fold from 0 to 3.5 h). If the initial ratio of the activity of CS239 to that of CS240 at time 0 is taken as 1, the ratio dramatically increased, reaching 5.8 at 3.5 h after temperature shift (Fig. 7B). In contrast, the lacZ gene without DB, pINZ, did not show any significant differences in its expression between CS240 and CS239, and the ratios of the activity of CS239 to that of CS240 remained almost at the initial level throughout the incubation time (Fig. 7B). A similar experiment was carried out with pINZDB3 (SD–, DB–), and the ratio of the activity in CS239 to that in CS240 increased 3.4-fold at 3.5 h after the temperature shift (data not shown). These results clearly demonstrate that the low levels of S2 protein at 42 °C causes significant stimulation of the lacZ expression only if the lacZ gene contains DB, consistent to the proposal of Shean and Gottesman (3).
DISCUSSION

The present results clearly demonstrate that there is a cis element downstream of the initiation codon, which in concert with SD plays an important role in the translation efficiency of mRNAs in E. coli. Our results provide supporting evidence for the DB hypothesis (1, 2) that DB forms a complex with the anti-DB in the 16 S rRNA to enhance translation initiation of DB-containing mRNA.

The present data reveal the following five features of the DB function. (a) The -galactosidase activity of the pJJG78DB2 is 2–3-fold higher than that of the pJJG78DB1, indicating that a better complementarity between DB and anti-DB yields better translation and/or that the first 3 residues (AUG) in the DB are required for the better activity of the DB. (b) The position of DB in the mRNA is quite flexible as DB can start from codon 1 (pINZDB1) or codon 6 (pINZDB4). (c) DB itself works very poorly in the absence of SD, suggesting that the formation of translation initiation complex could be first initiated by the SD-16 S rRNA interaction, which subsequently leads to the DB-anti-DB interaction. Alternatively, the SD-16 S rRNA interaction could be stabilized by DB-anti-DB base pairing. It has been shown that the 30 S ribosomal subunit can bind to mRNA in the absence of initiator tRNA to form an intermediate translation initiation complex (22). This intermediate complex could be stabilized by the interaction of DB with anti-DB. (d) DB enhances the translation initiation as judged by increased mRNA translational efficiency both in vivo and in vitro as observed by the increase of polysome formation and -galactosidase production in a cell-free system. (e) In the absence of S2 protein, the DB function is enhanced, consistently with the notion that anti-DB becomes more accessible to DB (3, 4). Since that the major conclusions for the behavior of the DB in translation have been made from artificially created DBs in overexpression systems as shown above, it would be important to analyze the effect of DB under more physiological conditions. In this regard, we have reported earlier that the DB is crucial for
the induction of the cold-shock protein A (CspA) at low temperature and, furthermore, that major cold-shock genes contain DB sequences (7). Moreover, we have recently postulated that the DB is essentially required for the induction of major cold-shock proteins under conditions completely blocking protein synthesis at low temperature (23). Future experiments testing artificially created DBs using single copies of DB-containing lacZ mRNAs would greatly support the conclusions about the role of the DB as a translational enhancer.

It should be noted that there are a number of mRNAs without the 5’-untranslated region such as lacI (3), mRNA from other phages (5), and Caulobacter crescentus (24). In these leaderless mRNAs, conclusive evidence indicates that DB plays an essential role in the formation of the translation initiation complex in the absence of SD (3). Such complexes in the absence of SD may be formed easier without any extra sequence upstream of the initiation codon (3, 24).

Among a number of cis elements in E. coli mRNAs known to enhance translation (25), DB next to SD appears to be found most often (6, 26–28). It is engaging to elucidate which of the two sequences (DB or SD) binds first to 30 S ribosomes for the formation of the initiation complex. In this regard, it should be noted that the SELEX method using 30 S ribosomes as a ligand resulted in enrichment of DB sequences accompanied with SD (8, 9).

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