Nonsense Mutations in cspA Cause Ribosome Trapping Leading to Complete Growth Inhibition and Cell Death at Low Temperature in Escherichia coli*

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CspA, the major cold shock protein of Escherichia coli, is dramatically induced immediately after cold shock. CspA production is transient and reduces to a low basal level when cells become adapted. Here we show that expression from multicopy plasmids of mutant cspA mRNAs bearing nonsense mutations in the coding region caused sustained high levels of the mutant mRNAs at low temperature, resulting in complete inhibition of cell growth ultimately leading to cell death. We demonstrate that the observed growth inhibition was caused by largely exclusive occupation of cellular ribosomes by the mutant cspA mRNAs. Such sequestration of ribosomes even occurs without a single peptide bond formation, implying that the robust translatability of the cspA mRNA is determined at the step of initiation. Further analysis demonstrated that the downstream box of the cspA mRNA was dispensable for the effect, whereas the upstream box of the mRNA was essential. Our system may offer a novel means to study sequence or structural elements involved in the translation of the cspA mRNA and may also be utilized to regulate bacterial growth at low temperature.

When an Escherichia coli culture growing at 37 °C is shifted to low temperature, cell growth stops temporarily. During this growth lag period called the acclimation phase, the synthesis of most cellular proteins is sharply reduced, whereas a specific set of proteins termed “cold shock proteins” are induced (1–4). Among these cold shock-inducible proteins, CspA, the major cold shock protein of E. coli (5), is induced to a level of 10^6 molecules/cell (6, 7), and its close homologues CspB, CspG, and CspI are also cold shock-induced to lesser extents (8–11). CspA folds into a five-stranded β-barrel structure and cooperatively binds to RNA and single-stranded DNA (12–14). It has been proposed to function as an RNA chaperone to facilitate translation or transcription antitermination at low temperature (14, 15). The CspA family is essential for E. coli cells to adapt to low temperature (11).

It has been shown that the cspA promoter is highly active at low temperature, even stronger than the lpp promoter, which is considered to be one of the strongest promoters in E. coli (16, 17). An AT-rich up-element immediately upstream of the −35 region has been implicated to contribute to the strength of the cspA promoter (18). The cspA promoter activity has also been shown to be modestly activated after cold shock (19, 20). At the second level, the cspA mRNA is stabilized by more than a hundred fold by a temperature downshift from 37 °C to 15 °C (16, 19).

In addition to the dramatic induction of the cspA mRNA upon cold shock, the mRNA is highly translatable at low temperature, whereas the translation of mRNAs for non-cold shock proteins is severely hampered in the acclimation phase. The exact mechanisms for the extraordinarily high translatability of the cspA mRNA have not yet been fully elucidated. However, in addition to its Shine-Dalgarno sequence, two other regions, the downstream box (DB) and the upstream box, have been proposed to play key roles in the translation initiation at low temperature (18, 21). These elements may enhance the ribosome recruitment either by directly interacting with the 30 S subunit or by directing a proper folding of the mRNA to optimize its interaction with 30 S ribosome subunits.

CspA production is intensely induced immediately after temperature drop, reaching its peak at approximately 1 h after temperature shift to 15 °C (5). Interestingly, when cell growth resumes after the acclimation phase, concomitantly the rate of CspA synthesis is significantly reduced to a low basal level. CspA has been implicated to be a negative regulator of its own gene expression, because in a strain with a deletion mutation of the cspA coding sequence the amount of the truncated cspA mRNA was much more than that in its parental strain, and the production of such truncated cspA mRNA was prolonged in the mutant cells (22).

Another intriguing observation is that when mutant cspA mRNAs that are unable to produce full-length CspA were overexpressed at low temperature, cell growth was completely inhibited (23). This phenomenon is termed the low temperature-dependent antibiotic effect of truncated cspA expression (LACE). Here, we show that the LACE caused by nonsense mutations in the cspA gene results from a nearly exclusive trapping of ribosomes by the mutant cspA mRNAs overexpressed at low temperature. Such trapping occurs even with a nonsense mutation at the second codon, indicating that the LACE can occur in the absence of translation elongation. This in turn suggests that translation initiation of the cspA mRNA is highly efficient and is responsible for its outstanding translatability.

* This work was supported by Grant GM19043 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: DB, downstream box; LACE, low temperature-dependent antibiotic effect of truncated cspA expression; UTR, untranslated region.
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**Fig. 1. Growth inhibition caused by mutant cspA expression.** A, construction of the cspA mutants. pJJG02 contains the full-length wild-type cspA gene as shown. The 159-base 5'UTR is shaded, and the thick black bar in the 5'UTR indicates the Shine-Dalgarno (SD) sequence. The striped box indicates the DB region. pA01S, pA10S, and pA30S were obtained by mutating the 2nd, 11th, and 31st codons of the cspA gene to TAA codons by site-directed mutagenesis using pJJG02 as a template. The black bars marked by asterisks show the positions of the TAA codons. B, growth curves of cold shocked cells harboring different plasmids. Note that cell cultures were diluted five times before A500 measurement. C, protein synthesis of cells harboring the different constructs after cold shock. The cells were pulse-labeled at 37°C at 1, 3, and 6 h after cold shock (CS). The position of CspA, a 7-kDa protein, is marked by an arrowhead. Other cold shock-inducible CspA homologues, CspB, CspG, and CspI also migrate at the same position as CspA. D, viability of cells harboring pA30S after cold shock. Circles show the viability of cells in liquid LB medium, and that on LB-agar plates is shown by squares.

**EXPERIMENTAL PROCEDURES**

*Bacteria Strains and Plasmids—E. coli strain TB1 (from New England Biolabs, Inc.), a hsdR (rR mK) derivative of JM83 (F’ ara Δ (lac-proAB) rpsL (Str r) [EasyTag Express protein labeling mix, 10 mM sodium phosphate buffer, pH 7.0, and then resuspended in 100 μl of SDS-protein sample buffer, boiled, and loaded on a 17.5% SDS-polyacrylamide gel (10 μl in each lane).*}

**RNA Extraction and Primer Extension—**RNAs were extracted from cells using the hot phenol method as described (25). The amounts of RNAs were quantified by their optical absorption at 260 nm, and their qualities were verified by agarose gel electrophoresis. Oligonucleotide APE (5' TTTTAGATACCATGCTAG 3') that is complementary to the coding sequence of the cspA mRNA was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and used as a primer. Primer extension reactions were performed for 1 h at 42°C using avian myeloblastosis virus reverse transcriptase in the presence of RNase Inhibitor (both from Roche Molecular Biochemicals). Two micrograms of RNA was used for each reaction. Primer extension products were resolved on a 6% denaturing polyacrylamide gel containing 6 M urea.

**Sucrose Density Gradient Fractionation of Ribosomes**—The cells were grown to an exponential phase (A500 = ~0.6–0.8) in 100 ml of LB medium and shifted to a 15°C water bath to start the cold shock treatment. At different time points after cold shock as indicated in the figures, chloramphenicol was added into cell cultures to a final concentration of 50 μg/ml, and 1 ml later cells were poured into prechilled centrifuge tubes containing 50 g of ice. The cells were pelleted by centrifugation for 10 min at 4°C, resuspended in 1 ml of ribosome buffer (20 mM Tris-Cl, pH 7.5, 50 mM NH4Cl, and 6 mM β-mercaptoethanol) containing 15 mM MgCl2 and 1 mg/ml lysozyme, and then stored at –80°C. The Cells were lysed by two rounds of freeze and thaw, and the lysates were then clarified by centrifugation for 15 min at 14,000 rpm in a microcentrifuge at 4°C. Cell lysates (400 μl each) were layered on 5–40% sucrose gradients made by ribosome buffer containing 10 mM MgCl2, and the gradients were centrifuged in a Beckman SW41 rotor at 35,000 rpm for 2.5 h at 4°C. After centrifugation the gradients were connected to a fast protein liquid chromatography system, fractionated, and recorded using the same system.

**Northern Blotting Analysis**—Cells harboring pA30S or pUC19 were cold shocked for 4 h and processed for sucrose gradient analysis. After centrifugation the gradient was fractionated into 0.5-ml fractions from the 50% sucrose interface and total RNA was extracted using the hot phenol method as described (25). The amounts of RNAs were quantified by their optical absorption at 260 nm, and their qualities were verified by agarose gel electrophoresis. Oligonucleotide APE (5' TTTTAGATACCATGCTAG 3') that is complementary to the coding sequence of the cspA mRNA was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and used as a primer. Primer extension reactions were performed for 1 h at 42°C using avian myeloblastosis virus reverse transcriptase in the presence of RNase Inhibitor (both from Roche Molecular Biochemicals). Two micrograms of RNA was used for each reaction. Primer extension products were resolved on a 6% denaturing polyacrylamide gel containing 6 M urea.
which RNAs were obtained by means of phenolchlorform (1:1) extraction and ethanol precipitation. Each RNA preparation (from 0.2 ml of each fraction) was dissolved in 20 µl of water. RNAs (3 µl each) were resolved on 1% agarose gels containing 1.5 M formaldehyde and then blotted onto a nylon membrane. The same blots were probed for cspA and lpp mRNA using their coding sequences as probes.

RESULTS

Growth Inhibition and Cell Death Caused by Nonsense Mutations in the cspA mRNA—To systematically study the LACE in a simplified system, we made point mutations in the cspA coding sequence at different positions to introduce nonsense mutations at the 2nd, 11th, and 31st codons into the wild-type cspA gene on plasmid pJJG02 (Fig. 1A). Cells harboring pJJG02 and the mutant plasmids were grown to a mid-log phase and subjected to cold shock treatment. As shown in Fig. 1B, the growth of cells harboring full-length wild-type cspA gene was recovered from an ∼1.5-h lag period in a similar manner as cells containing the vector pUC19 alone. On the other hand, cells harboring the mutant plasmids were unable to resume growth after temperature downshift. These cells were also unable to form colonies at 15 °C or 20 °C (not shown).

To see whether the growth inhibition was due to a block in protein synthesis, cells harboring different plasmids were pulse-labeled by [35S]methionine after cold shock. As shown in Fig. 1C, protein synthesis of cells harboring pUC19 and pJJG02 containing the full-length cspA gene was reduced temporarily (at 1 h) after cold shock but soon recovered (at 3 and 6 h). However, there was virtually no protein production in cells harboring any of the mutant plasmids after cold shock. It should be noted that even the production of cold shock proteins encoded by the chromosome (marked by an arrowhead) was significantly diminished in cells harboring the mutant plasmids. Note that no peptide is produced from pA01S because a nonsense codon exists next to the initiation codon, whereas small peptides consisting of 10 and 31 residues may be produced from pA10S and pA30S. These small peptides could not be seen on the present SDS-polyacrylamide gel.

Next we examined the viability of cells after cold shock. Cells harboring pA30S were withdrawn from a culture at different time points after cold shock and plated on agar plates after appropriate dilution. The plates were then incubated at 37 °C overnight, and the resulting colonies were counted. As shown in Fig. 1D, the LACE caused cell death with a half-life of 2 days. The cells lost viability at a faster rate, with a half-life of slightly longer than 1 day, when they were first plated on agar plates and then incubated at 15 °C (Fig. 1D). In both cases colonies formed by viable cells were found to be highly heterogeneous (not shown), suggesting that cellular damages caused by the LACE were not uniform.

Derepression of the Mutant cspA mRNA Production as a Result of Nonsense Mutations—Given the very small sizes of the peptides produced from the mutant plasmids, it is unlikely that the observed growth inhibition was caused by these gene products. In particular, pA01S having a nonsense codon immediately after the initiation codon is unable to produce any peptide. Therefore, it is assumed that the mutant mRNAs produced from the plasmids are responsible for the inhibition of cell growth. Thus, we next compared the ribosomal profiles of cold shocked cells harboring the mutant constructs with those harboring the wild-type cspA plasmid (pJJG02) or the vector plasmid (pUC19). At 1 h after temperature downshift, cells containing pUC19 had almost only the 70 S ribosomes with a few small polysome peaks on a sucrose gradient profile (not shown), indicating a block in translation after cold shock stress. On the other hand, cells containing pJJG02 showed a number of distinct polysome peaks (Fig. 3A), presumably as a result of active translation of the cspA mRNA transcribed from the plasmid.

Interestingly, in cells harboring pA01S, which contains no cspA open reading frame because of the termination codon immediately after the initiation codon created in pJJG02, no polysomes were formed except for the major peak of the 70 S ribosome and a small peak at the disome position (Fig. 3B). In cells harboring pA10S, which is able to encode a 10-residue peptide from a 30-base cspA coding sequence, a new major peak was formed at the disome position in addition to the one at the 70 S position (Fig. 3C). Because the coding sequence of pA30S was extended to 90 bases encoding a 30-residue peptide, yet another new major peak was formed at the trisome position in addition to those at the 70 S monosome and disosome positions (Fig. 3D). It is important to note that the same three major-peak profiles were maintained even after 24 h of cold shock (not shown). Thus, the number of polysome peaks observed is well correlated with the length of the cspA open reading frame retained in the individual mutant plasmid, strongly suggesting that most translating ribosomes in the cells are engaged in interacting with the mutant cspA mRNAs.

Trapping of Cellular Ribosomes by Mutant cspA mRNAs under the LACE—To prove that most cellular ribosomes are
indeed bound to the mutant cspA mRNAs, the association of the mutant cspA mRNAs and the non-cold shock lpp mRNA with ribosomes was examined using cells harboring pA30S. The transcript of lpp, the gene coding for a major outer membrane lipoprotein, was chosen to represent non-cold shock mRNAs. This choice was based on the following two reasons: first the gene is well expressed at both 37 °C and 15 °C, and second the mRNA is highly stable to withstand lengthy experimental procedures.

RNAs were extracted from every fraction of the sucrose gradient and subjected to Northern blotting analysis. In cold shocked cells harboring pA30S, over 90% of the cspA mRNA was found to be associated with ribosomes (Fig. 4A, nearly 94% in this particular experiment shown). Interestingly, when the same blot was probed for the lpp mRNA, the majority of this mRNA was found at the top of the gradient with less than 10% associated with the 70 S, and almost no signal was detected in the polysome fractions (Fig. 4A). In control cells harboring pUC19, virtually 100% of the lpp mRNA was detected in the fractions corresponding to the 70 S and polysomes (Fig. 4B). When the same filters were probed for the ompA mRNA, in pUC19 cells the mRNA was readily detected and mostly located in fractions corresponding to polysomes consisting of more than four 70 S units (data not shown), indicating that the mRNA was actively translated, whereas the mRNA was hardly detectable in cells harboring pA30S and absent in the polysome fractions (data not shown). In light of the observation that the amount of lpp mRNA, estimated by the necessary exposure time to achieve approximately equal signal intensity, also appeared to be less than that in control cells, it seems that the exclusion of these mRNAs from ribosomal protection greatly facilitated their degradation. These results demonstrate that the cspA mRNA is highly competitive in translation, effectively excluding the interaction of other cellular mRNAs with ribosomes.

The Essential Role of the Shine-Dalgarno Sequence and the Initiation Codon for the LACE—Next we attempted to prove that the translation initiation step is crucial for the LACE caused by the mutant cspA mRNAs. For this purpose, mutational analysis was carried out using pA03S containing a nonsense mutation (AAA to TAA) at the fourth codon of the cspA gene in pJJG02 (Fig. 5A). Cells transformed with pA03S were unable to form colonies at low temperature (Fig. 5C), and their growth in a liquid medium was also severely inhibited at low temperature (Fig. 5D). When either the SD sequence or the
AUG initiation codon of pA03S was abolished (pA03Sm1 and pA03Sm2, respectively; Fig. 5A), cells transformed with these plasmids became capable of forming normal-sized colonies at low temperature (Fig. 5C). Cells harboring pA03Sm1 grew in liquid LB medium at low temperature as normally as cells harboring pUC19, and those harboring pA03Sm2 also grew normally except for a longer lag period (Fig. 5D). It is important to note that the two above mutations did not affect the induction of the mRNA after cold shock (Fig. 5B). These results indicate that the normal ribosome association with mRNAs through the Shine-Dalgarno sequence and the initiation codon is required for the LACE.

Interestingly, the cspA mRNA contains a second AUG at the fifth codon, which is completely conserved among cold shock-inducible cspA homologues such as cspB, cspG, and cspI but not present in non-cold shock cspA homologous genes cspC, cspD, and cspE (2). Therefore, we examined the possible role of this AUG codon in the LACE by replacing it with a CUG (Leu) codon in pA03S (Fig. 5A). This mutation failed to rescue the cells from the LACE both on an agar plate and in a liquid medium at low temperature (Fig. 5C and D, respectively), implying that this codon may not play a role in the translation of the cspA mRNA at low temperature. Again, this mutation did not affect the amount of mRNA induced by cold shock (Fig. 5B).

The Downstream Box of the cspA mRNA Is Dispensable for the LACE—It has been proposed that the cspA mRNA contains a DB, a 15-base sequence downstream of its initiation codon and complementary to a part of the 16S ribosomal RNA (anti-DB), which functions as a translation enhancer at low temperature (18). To determine whether the DB is essential for the LACE, we generated a construct that had a deletion of the portion of the cspA coding sequence downstream of the DB to minimize its potential interference to either RNA structure or stability. Such a construct, pA10D, was made from pA03S by truncating the mutant cspA gene after the DB (Fig. 6A). The p-independent transcription terminator of cspA forming a stem-loop structure followed by a U-rich track (Fig. 6A) was then connected to the truncation point with three extra U residues. These three bases were added in an attempt to reduce possible steric hindrance to the potential interaction between the DB and the 30 S ribosomal subunit by the sizable stem-loop structure.

Cells with pA10D were unable to form colonies (not shown) and could not grow at all in a liquid medium at low temperature (Fig. 6B). Furthermore, these cells were found to lose viability after cold shock (not shown), similar to cells harboring pA03S. These results suggest that the DB may either indeed enhance the ribosome recruitment to this mRNA or in this situation simply provide enough space required for the ribosome to form the initiation complex with the mRNA. To distinguish the two possibilities and to determine whether the DB of the cspA mRNA is required for LACE, two mutants were constructed base on pA10D in an attempt to minimize the se-
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Previously, serial deletion analysis of the unusually long untranslated region (UTR) of the cspA mRNA has identified a 26-base-long sequence, named the “upstream box,” that is essential for the translation of the cspA mRNA (21). The central part of this sequence was also found to be complementary to the sequence from bases 1021 to 1035 of the 16 S ribosomal RNA (21). The upstream box, located immediately upstream of the Shine-Dalgarno sequence, is proposed to be essential for the mRNA to achieve the proper folding that facilitates ribosome binding. We therefore examined the role of the upstream box in the LACE.

Two upstream-box mutants of pA10D, pA10Dm4, and pA10Dm5 were constructed by deleting either the entire 26-base upstream box or its 13-base central region that is complementary to the 16 S ribosomal RNA (Fig. 8A). Cells transformed with the three plasmids were tested for cold sensitivity both on plates and in a liquid medium. Interestingly, three distinct phenotypes were observed. As mentioned before, cells transformed with pA10D were cold-sensitive both on plates and in a liquid medium, whereas cells with pA10Dm4 were able to form normal sized colonies (not shown) and grow in a liquid medium in an almost identical fashion to cells harboring pUC19. Cells with pA10Dm5, however, showed an intermediate phenotype, forming smaller colonies on plates (not shown) and growing in a liquid medium at a much reduced rate than the control cells (Fig. 8C). These cells were found to contain plasmid-derived cspA mRNAs at similar levels at 15 °C, suggesting that the deletion of the upstream box abolished the exceptional translatability of the pA10D mRNA; thus the mutant mRNAs lost their ribosome trapping capability.

**DISCUSSION**

In this study, we demonstrated that overexpression of mutant cspA mRNAs bearing nonsense mutations caused in most cases complete growth inhibition and cell death at low temperature. Consistent with our previous proposal that CspA negatively regulates its own gene expression, the nonsense mutations in cspA that abolished the production of CspA resulted in the derepression of the mutant mRNAs expressed from multicopy plasmids. Most of these mRNAs were found to be associated with ribosomes in the cells. On the other hand, the lpp mRNA, which under normal conditions was almost exclusively associated with ribosomes, became dissociated under the same condition. These results clearly demonstrate that the LACE is caused by the trapping and sequestration of cellular ribosomes by the mutant cspA mRNAs at low temperature.

Eradication of CspA production by the nonsense mutations appears to be the primary cause of the derepression of mutant mRNAs. In addition, the derepression may be enhanced by the insufficient induction of exoribonuclease polynucleotide phosphorylase, a cold shock protein likely to be responsible for clearing cold shock mRNAs at the end of acclimation phase (27), in LACE-affected cells. Furthermore, the tight packaging of ribosomes may also effectively protect the covered mRNAs from being degraded and thus contribute to the derepression of the mutant mRNAs. Consistent with this notion, it was found that the stability of a certain mRNA studied was correlated with its ability to trap ribosomes. For instance, pA03S mRNA capable of trapping ribosomes was well maintained after cold shock, whereas pA03Sm1 mRNA unable to bind ribosomes was efficiently degraded after the initial induction in the acclimation phase (Fig. 5B).

It is known that each ribosome occupies ~30–35 bases on a mRNA (26, 29). Therefore, it is conceivable that the mRNA from pA03S with a 50-base open reading frame can accommodate at most two ribosomes, one at the initiation codon and the other at the termination codon, whereas the mRNA from pA03S with a 90-base open reading frame can be occupied by three ribosomes. Indeed this notion was confirmed by the analysis of polysome patterns (Fig. 3). Therefore, under the LACE the upper limit of the size of polysomes is determined by the position of a nonsense codon in the cspA gene. The ribosome profiles in Fig. 3 also indicate that the ribosomes are tightly
stacked on the available coding regions of the mutant cspA mRNAs.

Among the LACE-causing plasmids, pA01S is particularly interesting, because the nonsense codon is placed immediately after the initiation codon in the cspA gene. Cells harboring pA01S contained almost only 70 S ribosomes after cold shock (Fig. 3B), yet the growth of these cells were completely inhibited (Fig. 1B), indicating that severe ribosome trapping occurred inside the cells. These results demonstrate that the robust translatability of the cspA mRNA is determined at the step of initiation and further imply that the formation of 70 S initiation complex with fMet-tRNA and pA01S mRNA outpaces the termination process at low temperature. The latter notion is also supported by the tight stacking of ribosomes on pA10S and pA30S mRNA. Considering that chloramphenicol might inhibit translation termination, we performed the polysome isolation without using chloramphenicol, and the result was essentially identical to the one obtained using the drug (data not shown). The slowness of translation termination may be further enhanced under the LACE, because the synthesis of certain factors required for the termination may be blocked in the absence of protein synthesis.

The first postulated translational enhancer of cold shock mRNAs has been the DB. β-Galactosidase fusion experiments showed that the proposed DB regions of the cspA and cspB mRNAs were essential for cold shock induction of the fusion proteins (18, 30). The initial hypothesis is that the DB promotes 30 S mRNA binding through its base pairing interaction with the anti-DB sequence of 16 S ribosomal RNA located in its penultimate stem (31, 32). Nevertheless, this model has been vigorously debated in recent years as the anti-DB is localized in the seemingly stable penultimate stem, and no such interaction has been detected (33, 34). A deletion of the anti-DB in the 16 S ribosomal RNA also did not significantly affect the DB-directed translation enhancement (35). Hence, the mechanism of the DB function remains elusive to date even though recent ribosome structures have assigned the anti-DB region exposed on the surface of the 30 S ribosomal subunit facing the 50 S subunit (36, 37), indicating that the DB of a mRNA could have access to the anti-DB if substantial melting of the helix occurs under certain conditions. Because the functionality of DB-like downstream sequences has been well established (Refs. 32, 38, and 39 and references therein), alternate working models remain to be explored to reconcile the above discrepancies. In the present study, mutation of the DB failed to rescue the LACE caused by pA10D. This might be caused by the overexpression of the mRNA from multicopy plasmids, because the potential contribution of the DB to the cspA mRNA translation could become dispensable when the mRNA amount reaches above a certain threshold. Therefore, any negative result obtained by the present system should be considered as inconclusive.

In the present study, we also demonstrated that in addition to the Shine-Dalgarno sequence and the initiation codon, the upstream box in the unusually long 5′-UTR of the cspA mRNA plays an important role in the formation of the translation initiation complex leading to the LACE. When the upstream box was deleted from pA10D, the resulted pA10Dm4 mRNA totally lost its translatability and failed to trap ribosomes, although the mRNA was constantly present, because cells appeared completely free from growth inhibition (Fig. 8). The role of the upstream box has been speculated to be involved in the mRNA folding to facilitate the ribosome-mRNA interaction (21), and the present results support this notion. The folding directed by the upstream box or the entire 5′-UTR may not be simply to expose the Shine-Dalgarno sequence and AUG codon to ease the formation of the initiation complex. More likely, it
may possess a certain RNA structural feature that could be recognized by either ribosomes or some other factors that facilitate ribosome recruitment.

Finally, it is also interesting to point out that the extent of the LACE, as judged by the severity of cell growth inhibition, caused by a certain mRNA seems to be well correlated with the efficiency of translation initiation on that mRNA. For example, cells with pA03Sm2 showed a long lag period before growth resumption (Fig. 5D). This result is fully consistent with the established principle that the SD plays a more important role in initiating ribosome binding than the AUG codon. Similarly, cells with pA10Dm4 grew faster than cells harboring pA10Dm5 (Fig. 8C), suggesting that a broader region of the upstream box is required for maximum translatability. Therefore, the present system may be used to further investigate elements in the cspA 5′-UTR that influence the rate and efficiency of translation initiation. The elucidation of the exact mechanisms may provide a new insight into developing a novel method to regulate or to completely block bacterial cell growth.

Acknowledgments—We thank Drs. S. Phadtare and R. Dutta for critical reading of the manuscript.

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