The Cold Box Stem-loop Proximal to the 5'-End of the *Escherichia coli* cspA Gene Stabilizes Its mRNA at Low Temperature*

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The 5'-end region of cspA mRNA contains a Cold Box sequence conserved among several cold-shock mRNAs. This region forms a stable stem-loop structure followed by an AU-rich sequence. Here we show that the Cold Box region is essential for the normal scale of cspA mRNA induction after cold shock because a deletion of the stem-loop significantly destabilizes the mRNA and reduces the cold shock-induced cspA mRNA amount by ~50%. The AU-rich track, however, slightly destabilizes the mRNA. The integrity of the stem is essential for the stabilizing function, whereas that of the loop sequence is less important. Overexpression of a mutant cspA mRNA devoid of both the AUG initiation codon and the coding region containing the 35°C termination causes the synthesis of CspA after cold shock, which is normally transient, to be substantially prolonged (14). This phenomenon is termed "derepression." In fact, overexpression of the first 25 bases is sufficient to cause the derepression of CspA expression after cold shock (14). Within the first 25 bases of cspA mRNA, a sequence element called the "Cold Box" is identified to be conserved among cspA and several other cold-shock mRNAs (14, 15). Deletion of the Cold Box from the 5'-UTR abolishes its derepression activity when overexpressed in trans and also appears to cause derepression in cis (16). Thus, it is postulated that the Cold Box is the binding site for a putative factor(s) that negatively regulates cspA expression and that overexpression of the 5'-UTR containing the Cold Box sequesters this factor so that cspA is released from the negative control and becomes constitutively expressed (16).

The major cold-shock protein of *Escherichia coli*, CspA, is dramatically induced upon an abrupt temperature downshift (1), with its abundance reaching approximately 1 million molecules/cell (2, 3). CspA induction is also triggered by certain antibotics (4) and by dilution of overnight cultures into fresh media, especially LB medium (2, 3). Eight other cspA homologous genes, cspB to cspI, have been identified in the *E. coli* genome; thus, *E. coli* contains a large cspA family consisting of nine members (5). CspA is not essential at both optimal and low temperatures (6), apparently as a result of substantial functional redundancy of CspA family members. Nevertheless, a ΔcspAΔcspBΔcspEΔcspG quadruple deletion strain is cold-sensitive and shows multiple defects at low temperature; therefore, the CspA family is indispensable for *E. coli* to adapt to cold-shock stress (7).

The mechanism of induced cspA expression has been studied extensively. It has been shown that the cspA promoter is highly active at both high and low temperatures (8). An AT-rich upstream-element immediately upstream of the −35 region has been implicated to contribute to the strength of the cspA promoter (9). It has also been reported that the cspA promoter is modestly activated after cold shock (10, 11). At the posttranscriptional level, cspA mRNA is extremely unstable at 37 °C and is stabilized by >100-fold by a temperature downshift to 15 °C (8, 10). It is thus presumed that the practically absent CspA expression in mid- to late exponential phase is attributable to the extreme instability of its mRNA and that the posttranscriptional mRNA stabilization plays a major role in the induction of CspA after cold shock. Finally, cold-induced cspA mRNA is well translated at low temperature.

Perhaps the most evident feature of the cspA mRNA is its long (159-nucleotide) 5'-untranslated region (UTR) (1). Alterations of this region profoundly affect the cspA mRNA level at 37 °C (8, 12). The same studies also show that the stability of the mRNA is influenced by manipulations on the 5'-UTR. There is also evidence suggesting that the 5'-UTR may have a role in regulating the transcription of the gene at 37 °C (12). Furthermore, a sequence element called the "upstream box" is essential for the translation of the mRNA at both high and low temperatures, indicating that the 5'-UTR is also involved in the translation of cspA mRNA (13).

Overexpression of the cspA 5'-UTR (followed by the 3'-UTR containing the transcription terminator) causes the synthesis of CspA after cold shock, which is normally transient, to be substantially prolonged (14). This phenomenon is termed "derepression." In fact, overexpression of the first 25 bases is sufficient to cause the derepression of CspA expression after cold shock (14). Within the first 25 bases of cspA mRNA, a sequence element called the "Cold Box" is identified to be conserved among cspA and several other cold-shock mRNAs (14, 15). Deletion of the Cold Box from the 5'-UTR abolishes its derepression activity when overexpressed in trans and also appears to cause derepression in cis (16). Thus, it is postulated that the Cold Box is the binding site for a putative factor(s) that negatively regulates cspA expression and that overexpression of the 5'-UTR containing the Cold Box sequesters this factor so that cspA is released from the negative control and becomes constitutively expressed (16).

The first 25 bases of cspA mRNA mentioned above form a stable stem-loop structure followed by an AT-rich track (Fig. 1A). A stable stem-loop structure at the 5'-end of an mRNA sometimes has an effect on the transcription of the gene, either causing RNA polymerase pausing or, on the contrary, preventing transcription arrest (17). In this study, we further investigated the role of this Cold Box region of cspA mRNA, taking it as a structural entity consisting of three parts: a helix of 6 bp, a stable stem-loop, and an AU-rich track.
a loop of 5 bases, and an AT-rich track of 6 bases. We found that the Cold Box stabilized the mRNA and that the stem was essential for this function. When an ORF-deleted cspa mRNA was overexpressed in the cell at low temperature, it was found to stably associate with ribosomes, suggesting that the 5'-UTR of the mRNA functions as a translational enhancer that is capable of binding ribosomes even in the absence of the AUG initiation codon and the coding sequence.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Media**—Strain HK01 was used for all Cold Box cis-effect experiments carried out using pBRWA-pCB09A and pMM158-pCB09Z plasmids. The strain was constructed by transducing a *penB* mutation from AR137 (MC1000 *ldhA-ompC* *penB*) (18) to a cspa/cspG double deletion strain BXX2 (JM38 *cspaA* *cspG*) (7) using P1 phage. The transductants were screened for tetracycline resistance (both from Roche Molecular Biochemicals). Two expression reactions were performed for 1 h at 30°C in plasmids pCBWT-09T, was used to detect RNAs transcribed from /H11032 which is complementary to the junction of the cspA 5'-UTR and 3'-GATTACAGGCTGAATTC-3'.

**RNA Extraction and Primer Extension**—Total cellular RNAs were extracted from cells harvesting pCBWT and pCB09T were cold-shocked for 4 h and processed for sucrose gradient analysis as described previously (20). After centrifugation, the gradients were fractionated into 0.5-mL fractions from which RNAs were obtained by means of phenol-chloroform (1:1) extraction and ethanol precipitation. Each RNA preparation (from 0.2 mL of each fraction) was dissolved in 20 μL of water, and 1 μL of each RNA was used in each primer extension reaction.

**mRNA Stability Measurement**—Cells harboring pBRWA, pCB01A, 08A, and 09A were cold-shocked at 15°C for 30 min, and then rifampicin was added to a final concentration of 200 μg/mL to stop transcription. Portions (1 mL) of cell cultures were taken at 0, 30, 60, and 90 min after the addition of rifampicin and subjected to RNA isolation. The amounts of cspa mRNA were subsequently determined by primer extension followed by Phosphorimage quantification.

**Pulse Labeling and SDS-PAGE**—Cells were grown in labeling medium to the exponential phase and shifted to a 15°C water bath. Portions (1 mL) of each culture were labeled with 5 μL of [55S]methionine (EasyTag Express protein labeling mix, 10 μCi/μL; PerkinElmer Life Sciences) for 5 min at 37°C or for 15 min at 15°C. Cells were chased by the addition of nonradioactive methionine to a final concentration of 5 mg/mL for 2 min at 37°C or 5 min at 15°C. Cells were washed with 20 mM sodium phosphate buffer (pH 7.0) and resuspended in 100 μL of SDS-protein sample buffer. Samples were boiled and resolved on 17.5% SDS-polyacrylamide gels (10 μL/lane). Gels were dried and exposed to x-ray films.

**RESULTS**

**Effect of Cold Box Deletion on Expression of the cspa Gene**—To further study the role of deletion of the Cold Box region on the expression of cspa, we first carried out a deletion analysis of the region by eliminating either the stem-loop, the AU-rich track, or both of them. Three mutant plasmids, pCB01A, pCB08A, and pCB09A, were generated by site-directed mutagenesis using pBRWA, which contains a full-length cspa gene on pBR322, as a template (Fig. 1A). In light of the potential role of the region in the induction of the cspa gene, we examined the amount of mRNA expressed from these four plasmids in an engineered *E. coli* strain, HK01, which is devoid of chromosomal cspa gene (as well as the cspG gene) and the penB gene encoding for a poly(A) polymerase (see “Experimental Procedures” for details). Because of the cspa deletion, the strain allows examination of plasmid-derived cspa mRNA without interference from the gene on the chromosome. Besides, the strain maintains pBR322-based plasmids at a very low copy number as a result of the *malT-ompB* gene encoding for a poly(A) polymerase.

HK01 cells transformed with the above-mentioned four plasmids were grown to the mid-exponential phase and subjected to cold-shock stress. After 0, 1, 2, 3, and 4 h of cold shock, total cellular RNAs were extracted, and the amounts of cspa mRNA were examined by primer extension analysis. Our previous hypothesis predicts that the deletion of the Cold Box results in prolonged and presumably increased expression of cspa mRNA. However, to our surprise, we found that the amount of the stem-loop deletion mutant mRNA (pCB01A) was ~50% lower than that of the wild-type mRNA (pCBWA) at 1 h after cold shock (Fig. 1B). A deletion of the AU-rich track (pCB08A) resulted in a slightly higher amount of mRNA upon cold shock, whereas a further deletion of the stem-loop (pCB09A) again caused an ~40% reduction of the mRNA amount at 1 h after cold shock (Fig. 1B). These results suggest that the stem-loop has an important role in regulating the cold-shock induction of cspa mRNA. Besides, the mRNAs expressed from all the above-mentioned plasmids were effectively reduced after 2 h of cold shock (Fig. 1B), indicating that the deletion of the Cold Box failed to cause any derepression at the level of the mRNA amount in cis under this condition.

**Destabilization of cspa mRNA by Deletion of the Stem-loop**—We reasoned that the decrease of the mRNA amount as a result of stem-loop deletion was caused by either a lower
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Fig. 1. Effect of Cold Box deletion on the cold-shock induction of the cspA gene. A, sequences and structures of the wild-type and deletion mutant mRNAs. pCBWA contains an intact Cold Box region consisting of a 6-bp stem, a 5-base loop, and a 6-base AU-rich sequence; pCB01A contains a deletion of the stem-loop; pCB08A has a deletion of the AU-rich track; and pCB09A has both the stem-loop and the AU-rich track deleted. The AU-rich region is shown in bold, and the deleted parts are shown as white boxes. B, cold-shock induction of the above-mentioned mutant cspA mRNAs. The top panel shows the amounts of different mutant cspA mRNAs at 0, 1, 2, 3, and 4 h after cold shock, as assayed by primer extension. The bottom panel shows the quantification of the results in A as determined by PhosphorImager.

It should be also noted that the extreme stability of cspA mRNA immediately after cold shock is transient and is progressively lost after cells become adapted to low temperature (10). At 2 h after cold shock, all four of these mRNAs became much more unstable as compared with 30 min after cold shock: the half lives of pCBWA, pCB01A, and pCB09A mRNAs became ~10 min, and that of pCB08A mRNA was slightly longer (data not shown). This is likely due to a recovery of certain nuclease activity after its initial cold-induced inactivation or an induction of cold shock-inducible nuclease activities such as polynucleotide phosphorylase, which has been proposed to be responsible for degrading cold-shock mRNAs after adaptation (22). CspA is an RNA chaperone and also negatively regulates expression of a certain ribonuclease such as RNase E, which may participate in the regulation of the stability of its own mRNA.

Effect of Mutations on the Stem-loop Structure—It appeared that the 5‘-stem-loop structure is important for the stability of cspA mRNA, which directly influences its induction/accumulation after cold shock. We further dissected the structure by making more mutations on this region. Six different mutations were generated on the structure, three on the stem and three on the loop, by site-directed mutagenesis (Fig. 3A). Then the amounts of the cspA mRNAs expressed from the above-mentioned mutant plasmids were examined by primer extension analysis. Quantification of the radioactive signals showed that all three mutations on the stem reduced the mRNA amount by 50–60% and that mutations on the loop either showed little effect (pCB05A) or reduced the mRNA amounts by ~30% (pCB06A and pCB07A, Fig. 3B). All the mutant cspA mRNAs were again found to be efficiently degraded after 3 h of cold shock, and no significant derepression effect was observed (Fig. 3B).

Effect of Cold Box Mutations on the Induction of a cspA-lacZ Fusion Reporter Gene—Because CspA regulates its own gene expression and may act on the Cold Box region (6, 14, 16), results obtained from mutations on the cspA gene may have been affected by its own gene product. Therefore, we next attempted to confirm the results presented above by examining the expression of a reporter gene in the absence of CspA. For this purpose, we constructed the same Cold Box region mutations as shown in Fig. 1 on a cspA-lacZ fusion construct, pMM158, in which the lacZ gene is fused after the thirteenth codon of the wild-type cspA gene on the plasmid. The fusion gene contains the entire cspA promoter together with the 5‘-UTR and shows cold shock-inducible β-galactosidase activity. We then measured the amounts of mRNAs produced from these mutants and the wild-type fusion gene by primer extension. It
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A

\[
\begin{array}{ccc}
\text{pBRWA} & \text{pCB02A} & \text{pCB03A} \\
\text{ACG-CAGU} & \text{ACG-CAGU} & \text{ACG-CAGU} \\
\text{A-U-U} & \text{A-U-U} & \text{A-U-U} \\
\text{U-A-U} & \text{U-A-U} & \text{U-A-U} \\
\text{U-A-U} & \text{U-A-U} & \text{U-A-U} \\
\text{G-C-U} & \text{G-C-U} & \text{G-C-U} \\
\end{array}
\]

B

![Graph showing the effect of Cold Box stem or loop mutations on the induction of cspA mRNA after cold shock.](image)

**Fig. 4. Effect of Cold Box mutations on the cold-shock induction of a cspA-lacZ fusion mRNA.** The top panel shows the amounts of fusion mRNAs of pMM158, pCB01Z, pCB08Z, and pCB09Z at 0, 1, 2, and 4 h after cold shock, as determined by primer extension. The bottom panel shows the relative mRNA amount quantified by a PhosphorImager.

was found that, consistent with the result obtained by mutational analysis on the authentic cspA gene (Fig. 1), deletion of either the stem-loop alone or the stem-loop plus the AU track reduced the cold-shock induction of the mRNA by ~50%, whereas deletion of the AU-rich sequence alone did not show any significant effect (Fig. 4). It is thus assumed that the decrease of mRNA amount as a result of Cold Box deletion was again due primarily to the destabilization of the mRNA. Both the wild-type and the mutant mRNAs appeared to be derepressed to a certain extent, presumably because of the lack of CspA, a negative regulator of its own gene, in those cells. β-Galactosidase activities of the constructs were also measured and found to correlate well with their mRNA amounts (data not shown), indicating that the Cold Box does not play a significant role in the translation of cspA mRNA.

**Effect of Cold Box Overexpression in trans**—It has been shown that overexpression in trans of the Cold Box-containing 5′-UTR of cspA mRNA results in derepression of the chromosomal cspA gene (14, 16). From the above-mentioned observations, it is speculated that the Cold Box is a binding site for a putative repressor that turns off the gene after the acclimation phase and that upon its overexpression it sequesters the repressor and therefore derepresses the chromosomal cspA gene. However, our results presented in this study seem to contradict this speculation. As a result, we attempted to confirm the previous finding by performing similar overexpression experiments and examining the effect on CspA production from the chromosome. The plasmid used in this study to overproduce the wild-type cspA 5′-UTR, pCBWT, contained a csaB sequence with its Shine-Dalgarno (SD) sequence and its open reading frame (together from +144 to +363) deleted (Fig. 5A). The remaining 5′-UTR was connected to the 3′-UTR by an EcoRI site and two G residues derived from vector pUC19.

As shown in Fig. 5B, the protein synthesis of cells carrying the control pUC19 plasmid dropped temporarily after cold shock (1 h, lane 2) and soon recovered (3 and 5 h, lanes 3 and 4, respectively), representing a typical cold-shock response and adaptation pattern. When a Cold Box-containing cspA mRNA lacking the coding region was overexpressed from pCBWT, the expression of the CspA family cold-shock proteins was indeed derepressed because CspA and its homologues were still expressed at 5 h after cold shock (Fig. 5B, lane 8). More interestingly, overall cellular protein synthesis was almost completely blocked, and even the expression of cold-shock proteins was significantly reduced (compare lane 6 with lane 2). A total of nine mutations identical to those previously shown (Figs. 1 and 3) were constructed based on pCBWT to examine the effect of the Cold Box in the overexpression system. When the entire Cold Box region is deleted (pCB09T; Fig. 5B, lanes 9–12), the protein synthesis pattern after cold shock became similar to that of pUC19 (lanes 1–4), indicating that the Cold Box does not play a significant role in the translation of cspA mRNA.

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FIG. 5. Effect of overexpression of ORF-less cspA mRNAs with Cold Box mutations on cspA expression and cellular protein synthesis after cold shock. A, schematic of pCBWT. This construct contains a cspA sequence with a deletion of region +144 to +363 encompassing the SD and coding sequence. +1 indicates the transcription start site (25), and the asterisk indicates the translation termination codon (TAA). The wild-type Cold Box is indicated as a thick black bar at the beginning of the 5′-UTR, and the downstream box (DB) is indicated as a hatched box in the coding sequence. The sequence containing an EcoRI site that connects the 5′-UTR and 3′-UTR is also shown. The 3′-UTR contains the factor-independent transcriptional terminator of cspA. B, protein synthesis of cells harboring vector pUC19, pCBWT, and various Cold Box mutants after cold shock. Cells harboring the indicated plasmids were pulse-labeled at 0, 1, 3, and 6 h after cold shock, and cellular proteins were then resolved by SDS-PAGE. The level of protein synthesis was visualized by autoradiography. The arrow indicates the position of CspA (other CspA homologues, CspB, CspE, CspG, and CspI, migrate at the same position).

FIG. 6. Effect of overexpression of ORF-less cspA mRNAs with Cold Box mutations on cell growth after cold shock. A, growth curve of cells expressing ORF-less cspA mRNAs after cold shock. B and C, amounts of plasmid-derived ORF-less cspA mRNAs at 0, 1, 3, and 7 h after cold shock, as determined by primer extension using a primer that specifically anneals to these RNAs.

Next, we measured the amounts of RNAs expressed from the different plasmids after cold shock by primer extension. It was found that cells harboring pCBWT (Fig. 6B, lanes 1–3) produced a large amount of plasmid-derived RNA, and notably, even at 7 h after cold shock, this RNA level was largely sustained (lane 3). In the case of pCB01T (Fig. 6B, lanes 4–6), in which the stem-loop of the Cold Box was deleted, the accumulation of their RNAs after cold shock was markedly reduced and also effectively repressed after initial induction. Deletion of the 6-base AU-rich sequence downstream of the stem-loop (Fig. 6B, pCB08T (lanes 7–9) and pCB09T (lanes 10–12)) caused the RNAs to be even slightly more sustainable than their counterparts, the pCBWT and pCB01T RNAs (Fig. 6B, compare lanes 9 and 3, lanes 11 and 5, and lanes 12 and 6). pCB02T (Fig. 6C, lanes 4–6) and pCB03T (Fig. 6C, lanes 7–9), which bear mutations on the stem, had an effect similar to that of pCB09T, whereas the loop mutants pCB08T and pCB06T (Fig. 6C, lanes 10–12 and lanes 13–15, respectively) behaved in a manner similar to that of the wild-type, pCBWT (Fig. 6C, lanes 1–3). These results support the conclusion that the stem of the Cold Box region has a more important function than the loop for the induction of cspA mRNA after cold shock. The significant reduction and effective repression of the amounts of RNAs produced from the stem-loop deletion and the stem sequence mutants as compared with the wild-type also strongly indicate that the stem-loop of the Cold Box effectively stabilizes cspA mRNA.

Association of the ORF-less cspA mRNAs with Ribosomes—To elucidate the mechanism by which overexpression of the ORF-less cspA mRNAs leads to growth inhibition, we studied the cellular localization of these RNAs. Cells containing pCBWT and pCB09T were collected 4 h after cold shock and lysed by rounds of freeze-thaw in the presence of lysozyme. Cell lysates were separated by sucrose gradient centrifugation and fractionated. RNAs were extracted from the fractions, and the plasmid-derived cspA RNAs were detected by primer extension.

Strikingly, primer extension analysis showed that the vast majority of the pCBWT RNA, although lacking both the SD and coding sequences, cofractionated with either the 30 S or 70 S ribosomes (Fig. 7A), indicating that the RNA was stably associated with these ribosomes. In the case of pCB09T RNA, which lacked the Cold Box, the total amount was evidently smaller than that of pCBWT RNA (compare the bottom panels of Fig. 7, B and A). Interestingly, this RNA still cofractionated with the 30 S and 70 S ribosomes, although a smaller fraction of its total

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Effect of overexpression of ORF-less cspA mRNAs with Cold Box mutations on cspA expression and cellular protein synthesis after cold shock. A, schematic of pCBWT. This construct contains a cspA sequence with a deletion of region +144 to +363 encompassing the SD and coding sequence. +1 indicates the transcription start site (25), and the asterisk indicates the translation termination codon (TAA). The wild-type Cold Box is indicated as a thick black bar at the beginning of the 5′-UTR, and the downstream box (DB) is indicated as a hatched box in the coding sequence. The sequence containing an EcoRI site that connects the 5′-UTR and 3′-UTR is also shown. The 3′-UTR contains the factor-independent transcriptional terminator of cspA. B, protein synthesis of cells harboring vector pUC19, pCBWT, and various Cold Box mutants after cold shock. Cells harboring the indicated plasmids were pulse-labeled at 0, 1, 3, and 6 h after cold shock, and cellular proteins were then resolved by SDS-PAGE. The level of protein synthesis was visualized by autoradiography. The arrow indicates the position of CspA (other CspA homologues, CspB, CspE, CspG, and CspI, migrate at the same position).
is assumed to lead to a prolonged translational block, and the ribosomes by a large amount of highly persistent pCBWT RNA (Fig. 7). The stable, unproductive binding of pCBWT RNA (data not shown). Contrary to our previous observation that deletion of the Cold Box led to derepression of cspA expression from the same gene, it was found that under the conditions used in the present study that the Cold Box had no such effect because the mutant cspA mRNAs with the Cold Box deleted were effectively cleared after 2–3 h of cold shock (Figs. 1, 3, and 6). Furthermore, we were unable to reproduce the previous in cis result. Taken together, our present results suggest that the Cold Box behaves mainly as an mRNA stabilizer, although its possible effect on transcription cannot be completely ruled out due primarily to the difficulty in directly measuring transcription activity in vivo.

With regard to the observation that a deletion of the Cold Box in the overexpression system largely abolished both chromosomal cspA derepression and cell growth inhibition at low temperature (Figs. 5 and 6), we consider the loss of growth inhibition as the primary outcome and the derepression as a secondary one. It is conceivable that the Cold Box deletion destabilizes the RNA induced after cold shock, leads to a sharp reduction of its amount, and therefore efficiently alleviates ribosome trapping, although the mutant RNA was still capable of ribosome binding (Fig. 7B). Because all the overexpression results can be explained by mRNA stability and ribosome sequestration, it becomes less likely that the Cold Box is a binding site for a putative factor negatively regulating the transcription of cspA.

The next question is why the pCBWT RNA, which lacks both the SD and the coding sequences, is able to interact with ribosomes. A closer examination revealed that although the original cspA SD sequence was deleted, an alternative one (GGA) was formed by the newly created EcoRI sequence in conjunction with its immediate upstream sequence (Fig. 5A). It should also be pointed out that this new SD sequence is located at the level of translation because little [35S]methionine incorporation was detected by a pulse labeling experiment (pCBWT; Fig. 5B). The overproduced ORF-less cspA mRNA appears to sequester a factor(s) essential for cell growth or protein synthesis rather than a regulatory factor for cspA transcription as previously speculated (16).

We showed that the 30 S and 70 S ribosomes were among the factors bound and potentially sequestered by the overexpressed pCBWT RNA (Fig. 7). The stable, unproductive binding of ribosomes by a large amount of highly persistent pCBWT RNA is assumed to lead to a prolonged translational block, and the derepressed production of CspA, a cold-shock RNA chaperone thought to enhance translation, is then likely to be a compensatory response of E. coli cells aimed at counteracting the translation inhibition. This is highly reminiscent of the observation that certain ribosome-targeting antibiotics, such as chloramphenicol, can induce the expression of cspA (4, 23) and also resembles the induction of cold-shock proteins after a temperature downshift, which is considered to lead mainly to a translational block as a result of potential ribosome defects as well as the stabilization of mRNA secondary structures.

The mechanism of the CspA derepression may be a stabilization of the chromosomal cspA transcripts under the growth inhibition condition, similar to that of the cold-shock induction of CspA. To this end, we have evidence that the chromosomal cspA mRNA is stabilized 3- to 4-fold when cell growth is inhibited at low temperature by a plasmid practically identical to pCBWT. Because all the overexpression results can be explained by mRNA stability and ribosome sequestration, it becomes less likely that the Cold Box is a binding site for a putative factor negatively regulating the transcription of cspA.

DISCUSSION

In this study, we demonstrate that the Cold Box stabilizes cspA mRNA during the acclimation phase initially after cold shock (Fig. 2). Consistent with previous observations, we found that overexpression of the 5'-UTR of cspA mRNA in trans resulted in the derepression of chromosomal CspA synthesis after cold shock (Fig. 5). It should be emphasized that overexpression of the wild-type 5'-UTR, while causing the derepression of CspA synthesis, also resulted in severe inhibition of cell growth (pCBWT; Fig. 6A). The growth inhibition was probably at the level of translation because little [35S]methionine incorporation was detected by a pulse labeling experiment (pCBWT; Fig. 5B). The overproduced ORF-less cspA mRNA appears to sequester a factor(s) essential for cell growth or protein synthesis rather than a regulatory factor for cspA transcription as previously speculated (16).

We showed that the 30 S and 70 S ribosomes were among the factors bound and potentially sequestered by the overexpressed pCBWT RNA (Fig. 7). The stable, unproductive binding of ribosomes by a large amount of highly persistent pCBWT RNA is assumed to lead to a prolonged translational block, and the
tiate the initial interaction or stabilize the binding complex at a later stage. Certainly, the AUG is essential for the initiation of protein synthesis.

In an effort to identify potential Cold Box-binding proteins using a Cold Box affinity column, we isolated a number of proteins, two of which have been identified as 30 S ribosome proteins. These proteins may bind the Cold Box in the cell and protect cspA mRNA from its degradation by either a 5′→3′ exonuclease or a certain endonuclease. At the same time, significant binding or sequestration of these proteins by Cold Box overexpression is likely to impede ribosomal assembly or function, contributing to cell growth inhibition. Further identification of such proteins and characterization of their interaction with the Cold Box are currently under way in the laboratory.

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The Cold Box Stem-loop Proximal to the 5′-End of the *Escherichia coli cspA* Gene Stabilizes Its mRNA at Low Temperature

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