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Role of the Cold-Box Region in the 5′ Untranslated Region of the *cspA* mRNA in Its Transient Expression at Low Temperature in *Escherichia coli*

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Upon temperature downshift, a group of proteins called cold shock proteins, such as CspA, CspB, and CsdA, are transiently induced in *Escherichia coli*. However, when the 5′ untranslated region (5′ UTR) of *cspA* mRNA is overproduced at low temperature, the expression of cold shock genes is prolonged or derepressed. It has been proposed that this effect is due to highly conserved 11-base sequences designated the "cold box" existing in the 5′ UTRs of *cspA*, *cspB*, and *csdA*. Here, we demonstrate that the overproduction of the 5′ UTR of not only *cspA* but also *cspB* and *csdA* mRNAs causes derepression of all three genes at the same time. Conversely, when the cold-box region was deleted from the *cspA* 5′ UTR its derepression function was abolished. The amount of mRNA from the chromosomal *cspA* gene was much higher in cells overproducing the wild-type 5′ UTR by means of a plasmid than it was in cells overproducing the cold-box-deleted 5′ UTR. The stability of the chromosomal *cspA* mRNA in cells overproducing the wild-type 5′ UTR was almost identical to that in cells overproducing the cold-box-deleted 5′ UTR. Therefore, the derepression of *cspA* caused by overproduction of 5′ UTR at the end of the acclimation phase occurs at the level of transcription but not by mRNA stabilization, indicating that the cold-box region plays a negative role in *cspA* transcription in cold shock-adapted cells. The role of the cold-box region was further confirmed with a *cspA* mutant strain containing a cold-box-deleted *cspA* gene integrated into the chromosome, which showed a high level of constitutive production of CspA but not CspB during exponential growth at low temperature.

When an exponentially growing culture of *Escherichia coli* is shifted from 37 to 15°C, there is usually a growth lag period, termed the acclimation phase, before the cells resume growth. A number of cold shock proteins are transiently induced during this phase (13). In addition to CspA, a major cold shock protein in *E. coli* (7), CspB (16), CspG (19), CsdA (14), and RbfA (15) have been shown to be cold inducible. CspA consists of 70 amino acid residues (7), and its three-dimensional structure, which is composed of five antiparallel β strands forming a β-barrel structure, has been determined by both X-ray crystallography (23) and nuclear magnetic resonance (20). Two RNA-binding motifs have been identified on β2 and β3 strands. CspA has been shown to function as an RNA chaperone at low temperature (10).

It has been demonstrated that parallel to the cold shock induction of CspA protein, the *cspA* transcript was highly induced upon temperature downshift, suggesting that *cspA* induction at low temperature may be regulated at the level of transcription (24). However, recently it has been found that the *cspA* transcript was substantially stabilized upon temperature downshift, and this stabilization plays a major role in the cold shock induction of *cspA* (2, 4, 6). Three base substitutions near the Shine-Dalgarno sequence caused a 150-fold enhancement of *cspA* mRNA stability, resulting in constitutive expression of *cspA* at 37°C, indicating that the *cspA* promoter is also active at high temperature (4). To what extent the *cspA* induction at low temperature is regulated at the level of transcription remains to be elucidated. In addition to regulation at the levels of transcription and mRNA stability, *cspA* expression at low temperature is also controlled at the level of translation, with the downstream box in the *cspA* coding sequence (18).

Also, it has been shown that overproduction of the 143-base sequence from base +1 to base +143 of the 159-base 5′ untranslated region (5′ UTR) of the *cspA* mRNA upon cold shock resulted in prolonged synthesis of not only CspA but also CspB and CsdA (9). All the mRNAs for these proteins contain unusually long 5′ UTRs (159, 161, and 226 bases for *cspA*, *cspB*, and *csdA*, respectively), and within each of these 5′ UTRs there is a highly conserved 11-base motif called the "cold box," which has been proposed to be a repressor binding site. In this report we demonstrate that overproduction of the 5′ UTRs of both *cspB* and *csdA* mRNAs can also cause the prolonged synthesis of CspA, CspB, and CsdA or simultaneous derepression of *cspA*, *cspB*, and *csdA* expression at the end of the acclimation phase. Deletion of the cold-box region abolished the derepression function of the 5′ UTR of *cspA* mRNA. Furthermore, the amount and stability of mRNA from the chromosomal *cspA* gene were examined by primer extension experiments to demonstrate that the derepression occurs at the level of transcription. The results provide further evidence to support the proposal that a putative repressor binds to the cold-box regions in the mRNAs for cold shock genes, which in turn blocks or attenuates the transcription of these genes (6).

MATERIALS AND METHODS

Strains and culture media. *E. coli* CL83 [*recA ara* (*lac-proAB*) *rpsL*(=*strA*)φ80 *lacZ M15*] (17) was used for all experiments and was grown in M9-Casamino Acid medium as described previously (8). For pulse-labeling experiments, an amino acid mixture lacking methionine was used. The final concentration of each amino acid was 50 μg/ml.

Plasmid construction. p6mTEK and p2JTEK were constructed as previously described (9).

The plasmid plmcsdA was constructed as follows. Primer 6724 (5′ TTGGTA CCTCCTGGGCCAGGACC 3′) and primer 6725 (5′ TCGAATTCGTAGTAC GTGTGCCT 3′) were used for PCR with a plasmid DNA containing wild-type

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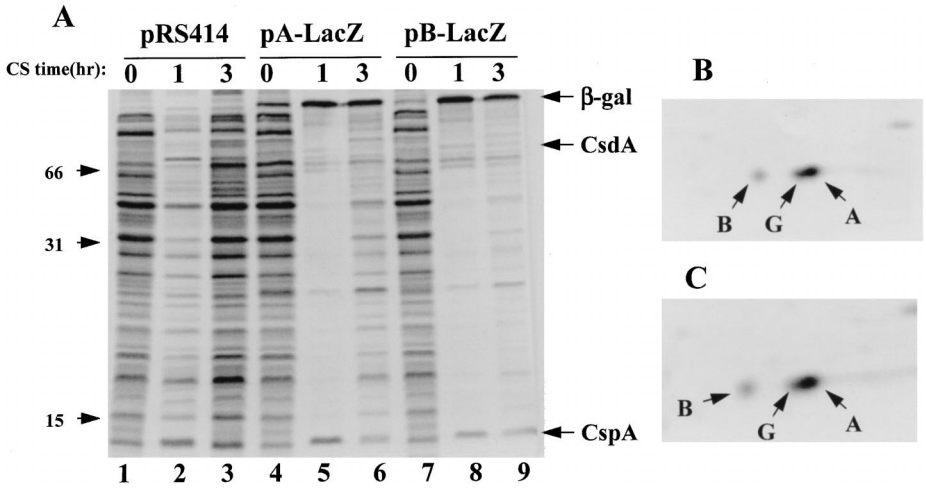

FIG. 1. Effects of overproduction of the 5′ UTRs of *cspA* and *cspB* mRNAs. Pulse-labeling experiments were carried out as described in Materials and Methods. (A) Cultures of cells harboring different plasmids (pRS414, pA-LacZ, and pB-LacZ) were shifted from 37 to 15°C at mid-log phase (80 Klett units). The time point (CS time, time after cold shock) of pulse-labeling is shown above each lane. The same amount of the culture (0.15 ml) was analyzed at each time point by SDS–17.5% polyacrylamide gel electrophoresis. The labeling times at 37 and 15°C were 5 and 15 min, respectively. The positions of CspA, CsdA, and β-galactosidase (β-gal) are indicated by arrows. Lanes 1 to 3, CL83 cells harboring the vector only; lanes 4 to 6, CL83 cells harboring pA-LacZ; lanes 7 to 9, CL83 cells harboring pB-LacZ. Molecular mass (in kilodaltons) is shown on the left. (B) Cells with pA-LacZ 3 h after cold shock (panel A, lane 6) were analyzed by two-dimensional gel electrophoresis. Only a portion of the autoradiogram is shown. Positions of CspA, CspB, and CspG are indicated by arrows. (C) Cells with pB-LacZ 3 h after cold shock (panel A, lane 9) were analyzed as described for panel B.

csdA as the template. The PCR product was then digested with *Eco*RI and *Kpn*I and ligated into p6mT digested with *Eco*RI and *Kpn*I. The resulting plasmid, plmcsdA, contained the 5′ UTR of the *csdA* mRNA under the *cspA* promoter, followed by the *cspA* transcription terminator sequence.

The plasmid pΔ24T was constructed by two-step PCR. For the first PCR, PCRI was produced with primer 3552 (5′ GACAGGATTAAAAATCGATG 3′) and primer 6727 (5′ CCTTACTACACTGCTCGTTGATGTGTGCATTA 3′) and PCRII was produced with primer 6726 (5′ AGCAGTGTAGTAAGG 3′) and primer 3550 (5′ TAATTAAGTGTGCCTTTCGG 3′). pJJG02 was used as the template in both PCRs. The second PCR was carried out with primers 3552 and 3550, using PCRI and PCRII as the templates. The final PCR product was then ligated into the *Sma*I site of pUC9. The *cspA* transcription terminator sequence was PCR amplified with primers 6290 (5′ CGGAATTCAGCCTGTA ATCTCT 3′) and 4860 (5′ CTGTCGACTTACTTACGGCGTTGC 3′). The PCR product was then digested with *Eco*RI and ligated into the plasmid described above, which was digested with *Eco*RI and *Ssp*I. This plasmid contained the 5′ UTR of *cspA* mRNA from base +1 to base +143, with a 22-base deletion from base +3 to base +24 under the *cspA* promoter. The DNA sequence of all the PCR products was confirmed by DNA sequencing (21).

The plasmid pKY701*cspA*(ΔCB) was constructed as follows. For the first PCR, PCRI was produced with primer 3552 and primer 6727 (see above) and PCRII was produced with primer 6726 and primer 4860 (see above), using pJJG02 as the template for both PCRs. The second PCR was carried out with primers 3552 and 4860, using PCRI and PCRII as the templates. The final PCR product was then ligated into the *Eco*RV site of a TA vector (Invitrogen Corp.). After the DNA sequence was confirmed, the 776-bp *Hin*dIII-*Sma*I fragment from the plasmid containing the cold-box-deleted *cspA* gene [*cspA*(ΔCB)] was inserted into pKY701 digested with *Hin*dIII-*Sma*I. pKY701 (26) is a plasmid which contains a temperature-sensitive replication origin [*ori*(Ts)] as well as the *lpp-28* gene as the plasmid integration site. The resulting plasmid was designated pKY701*cspA*(ΔCB).

Pulse-labeling experiments. The pulse-labeling experiments were carried out as described previously (8). Proteins were analyzed by sodium dodecyl sulfate (SDS)–17.5% polyacrylamide gel electrophoresis (8). Two-dimensional gel electrophoresis was carried out as previously described (12).

Primer extension experiments. *E. coli* CL83 harboring different plasmids was grown to mid-log phase at 37°C and then shifted to 15°C for 3 h. Total RNA was extracted by the hot-phenol method (22). Primer extension experiments were carried out with avian myeloblastosis virus reverse transcriptase (Boehringer) according to the method of Gafny et al. (5). The following two primers were used: primer 3551 (5′ TTTAGAGCCATCGTCAGGAG 3′), complementary to the sequence from base +243 to base +224, and primer 4592 (5′ GTGCACTA CGAGGGGTATCA 3′), complementary to the sequence from base +82 to base +63.

mRNA stability. *E. coli* CL83 harboring different plasmids was grown to mid-log phase at 37°C and then shifted to 15°C. After 3 h of incubation at 15°C, rifampin was added to a final concentration of 200 μg/ml. At 0, 5, 10, 20, and 30 min after the addition of rifampin, 1.5 ml of the culture was taken for RNA extraction by the hot-phenol method (22). Primer extension experiments were carried out with primer 3551.

Chromosomal integration of cold-box-deleted *cspA*. Plasmid pKY701*cspA* (ΔCB), which contains a temperature-sensitive replication origin [*ori*(Ts)], was introduced into strain WB002, a Δ*cspA* strain (4), and plated out at 42°C. From a colony formed at 42°C a single colony was reisolated at 42°C. Using the chromosomal DNA from this isolate, we confirmed the integration of the plasmid into the WB002 chromosome by PCR. The resulting strain was designated WB003.

RESULTS

Effects of overproduction of 5′ UTRs of *cspA*, *cspB*, and *csdA* mRNAs. First, we attempted to test the effect of overproduction of the 5′ UTR of the *cspB* mRNA as well as that of the *csdA* mRNA on the expression of cold shock genes at the end of the acclimation phase. In order to overproduce the 5′ UTR of the *cspB* mRNA, pB-LacZ, in which the *cspB* gene was fused with the *lacZ* gene in frame at the 13th codon (3), was used. Note that the plasmid was a derivative of pBR322, whose copy number is considered to be approximately 30 to 50 per cell, and that this copy number was adequate to exert the derepression effect on cold shock genes (9). As a positive control, pA-LacZ, in which the *cspA* gene was fused with the *lacZ* gene in frame at the 13th codon (3), was used. *E. coli* CL83 cells transformed with these plasmids were used for pulse-labeling experiments as shown in Fig. 1. Cells were labeled for 15 min at 15°C 0, 1, and 3 h after cold shock. Cells transformed with the vector pRS414 were used as another control for the pulse-labeling experiments. CspA expression reached the maximum level 1 h after cold shock (Fig. 1A, lane 2) and dropped to a new basal level 3 h after cold shock (Fig. 1A, lane 3) in the cells carrying the vector pRS414 alone. In contrast to the control cells, in cells carrying pA-LacZ, high levels of production of CspA, CspB, and CspG (all of which migrated at the same position [see below]), as well as CsdA (at 70 kDa), were still maintained at 3 h (Fig. 1A, lane 6), confirming the previous results of Jiang et al. (9). With the cells carrying pB-LacZ, the same expression patterns of CspA, CspB, CspG, and CsdA were observed (Fig. 1A, lanes 7, 8, and 9), as with the cells carrying pA-LacZ (Fig.

1A, lanes 4, 5, and 6). Since CspA, CspB, and CspG migrate at the same position in SDS-polyacrylamide gel electrophoresis, the samples with pA-LacZ and pB-LacZ were also analyzed by two-dimensional gel electrophoresis. All three proteins, CspA, CspB, and CspG, were still produced 3 h after temperature downshift for both cells with pA-LacZ (Fig. 1B) and cells with pB-LacZ (Fig. 1C), indicating that not only *cspA* but also *cspB* and *cspG* were derepressed when the 5′ UTR of *cspA* or *cspB* was overproduced. These results demonstrate that overproduction of the 5′ UTR of not only the *cspA* mRNA but also the *cspB* mRNA causes the prolonged expression or derepression of *cspA*, *cspB*, *cspG*, and *csdA* at the end of the acclimation phase. In these experiments, it is interesting to point out that as cold shock gene expression at 3 h after cold shock is derepressed, the synthesis of other cellular proteins is not fully recovered to the level of control cells (compare Fig. 1A, lanes 6 and 9 for cells carrying pA-LacZ and pB-LacZ, respectively, with lane 3 for the control cells), suggesting that overproduction of not only the *cspA* 5′ UTR, as shown previously (9), but also the *cspB* 5′ UTR causes a negative effect on the expression of non-cold shock genes.

Next we examined the effect of overproduction of the 5′ UTR of the *csdA* mRNA on the expression of cold shock genes at low temperature. For this purpose, the 226-bp DNA sequence corresponding to the 5′ UTR of *csdA* (from base +1 to base +226, with the transcription start site defined as +1) (25) was placed under the control of the *cspA* promoter. At the 3′ end, the *cspA* transcription terminator sequence was also added to secure transcription termination. The resulting plasmid, designated plmcsdA, was introduced into CL83 cells, and pulse-labeling experiments were then carried out with the transformed cells. As shown in Fig. 2, overproduction of the 5′ UTR of *csdA* mRNA also caused derepression of *cspA* (and *cspB* and *cspG*) as well as *csdA*. A high level of production of CspA and CsdA (Fig. 2, lane 3) was observed 3 h after cold shock with concomitant reduction of the synthesis of non-cold shock proteins, as observed with pA-LacZ and pB-LacZ, indicating that overproduction of the 5′ UTR of *csdA* mRNA is capable of causing the same effect as overproduction of those of *cspA* and *cspB* mRNAs.

The level of derepression of *cspA* and *csdA* 3 h after cold shock was much higher in cells carrying plmcsdA (Fig. 2) than in cells carrying pA-LacZ and pB-LacZ (Fig. 1), while the synthesis of non-cold shock proteins was more severely inhibited (compare Fig. 2, lane 3 to Fig. 1A, lanes 6 and 9). This is likely due to the fact that the vector used for plmcsdA was pUC9, while pA-LacZ and pB-LacZ were pBR322 derivatives.

Effects of the deletion of the cold-box region. The derepression of cold shock genes caused by overproduction of the 5′ UTRs of *cspA*, *cspB*, and *csdA* mRNAs at the end of the acclimation phase is considered to be due to a highly conserved sequence consisting of 11 bases (the cold box) (9). The cold-box consensus sequence is UGACGUACAGA, and there is only one base mismatch between *cspA* and *csdA* and two base mismatches between *cspA* and *cspB* in this region. The cold box is considered to be responsible for the derepression effect of the 5′ UTRs of *cspA*, *cspB*, and *csdA* mRNAs. We next tested the effects of overproduction of mRNAs with and without the cold-box region on the expression of cold shock genes. For this purpose, pΔ24T was constructed, which overproduces a 5′ UTR of the *cspA* mRNA encompassing the region from base +1 to base +143 but containing a deletion of the cold-box region from base +3 to base +24. CL83 cells were transformed with the plasmid, and pulse-labeling experiments were carried out. Cells were labeled for 15 min 3 h after cold shock. Experiments were also carried out with cells transformed with

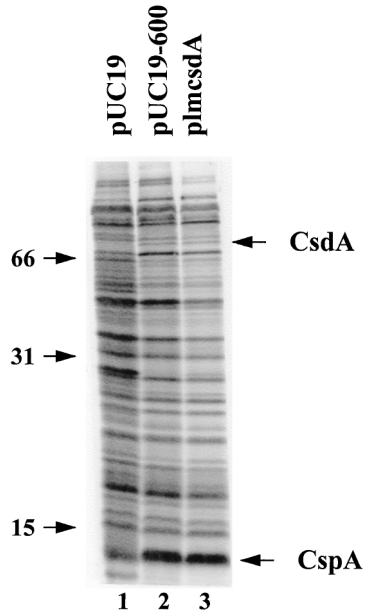

FIG. 2. Effects of overproduction of the 5′ UTR of *csdA* mRNA. Cultures of cells harboring the indicated plasmids were shifted from 37 to 15°C at mid-log phase (80 Klett units) and incubated at 15°C for 3 h. Cells were then labeled with [^{35}S]methionine for 15 min as described in Materials and Methods. The same amount of the culture (0.15 ml) was used for each labeling experiment and analyzed by SDS–17.5% polyacrylamide gel electrophoresis. Lane 1, CL83 cells harboring the vector pUC19; lane 2, CL83 cells harboring pUC19-600 for overproduction of the 5′ UTR of *cspA* mRNA; lane 3, CL83 cells harboring plmcsdA for overproduction of the 5′ UTR of *csdA* mRNA. Positions of CspA and CsdA are indicated by arrows. Molecular mass markers (in kilodaltons) are shown on the left.

p6mTEK, which overproduces only the first 6-base sequence of the *cspA* mRNA, and p2JTEK, which overproduces the first 25-base sequence of the *cspA* mRNA, including the cold box. As shown in Fig. 3, lanes 1 and 3, both p6mTEK and pΔ24T lost the derepression function while p2JTEK was still capable of derepressing cold shock gene expression (Fig. 3, lane 2), indicating that the cold-box region is responsible for the derepression effect.

Derepression is caused at the level of transcription. *cspA* expression is normally repressed to a new basal level after 3 h of incubation at 15°C, while overproduction of the 5′ UTR of *cspA* mRNA results in failure of the repression of *cspA*, causing a high level of CspA production 3 h after cold shock (9). In order to elucidate whether this high level of *cspA* expression is caused at the level of transcription or of mRNA stabilization, we next attempted to determine the amount of *cspA* mRNA in cells 3 h after cold shock. For this purpose, CL83 cells were transformed with pΔ24T and pUC19-600 (9), which contains the 5′ UTR from base +1 to base +143 of *cspA* mRNA under the control of the *cspA* promoter. Total RNA was extracted from the cells after 3 h of incubation at 15°C and used for primer extension experiments with the following two primers. The first primer (4592) was complementary to the sequence from base +82 to base +63 of *cspA* mRNA, which is capable of detecting the transcripts from both the plasmid and the chromosome. The second primer (3551) was complementary to the sequence from base +243 to base +224, corresponding to a region in the coding sequence of *cspA*. Thus, this primer detects transcripts only from the chromosomal *cspA* gene. As shown in Fig. 4, lane 4, primer 4592 detected a large amount of transcript from pΔ24T while the amount of transcript from the

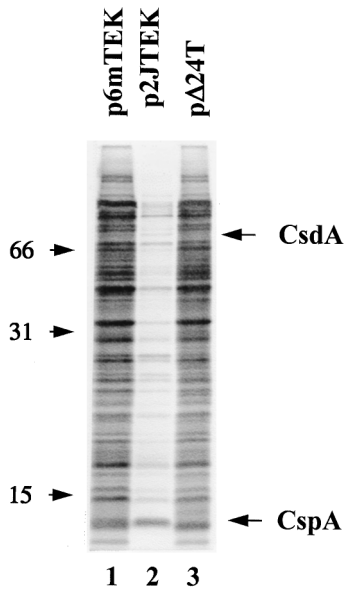

FIG. 3. Effects of deletion of the cold-box region. Pulse-labeling experiments were carried out as described in Materials and Methods. Cells harboring p6mTEK, p2JTEK, and pΔ24T were shifted from 37 to 15°C at mid-log phase (80 Klett units) and then labeled with [^{35}S]methionine for 15 min 3 h after cold shock. The plasmids were used to overproduce *cspA* mRNA as follows: p6mTEK from bases +1 to +6, p2JTEK from bases +1 to +25, and pΔ24T from bases +1 to +143 with a deletion of the cold-box region from +3 to +24. Protein expression patterns were analyzed by SDS–17.5% polyacrylamide gel electrophoresis. Lane 1, CL83 cells harboring p6mTEK; lane 2, CL83 cells harboring p2JTEK; lane 3, CL83 cells harboring pΔ24T. The positions of CspA and CsdA are indicated by arrows. Molecular mass markers (in kilodaltons) are shown on the left.

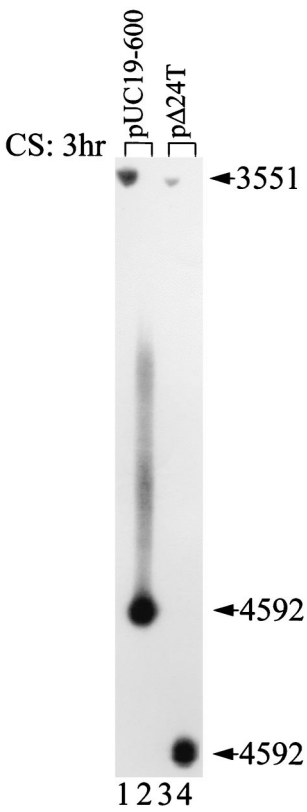

FIG. 4. Detection of *cspA* transcripts in cells overproducing the 5′ UTR of *cspA* mRNA (from bases +1 to +143) with and without the cold-box region from bases +3 to +24. Total RNA was isolated from CL83 cells carrying pUC19-600 (control; lanes 1 and 2) and pΔ24T (cold-box deletion; lanes 3 and 4) after 3 h of incubation at 15°C. Primer extension experiments were carried out as described in Materials and Methods. Two primers were used in order to detect the *cspA* transcript from the chromosomal *cspA* gene (primer 3551; lanes 1 and 3) and the transcripts from both plasmids and the chromosome (primer 4592; lanes 2 and 4). The products of primer extension were separated by 7 M urea–6% acrylamide gel electrophoresis. The positions of the products of the different primers are indicated by arrows. CS: 3 hr, 3 h after cold shock.

chromosomal *cspA* gene detected by primer 3551 (Fig. 4, lane 3) was very small, corresponding to the low production of CspA in the cells harboring pΔ24T after 3 h at 15°C (Fig. 3, lane 3). In contrast, the amount of transcript from the chromosomal *cspA* gene in the cells harboring pUC19-600 (Fig. 4, lane 1), as detected by primer 3551, was much greater than that in the cells harboring pΔ24T (Fig. 4, lane 3). Note that a large amount of the transcript from pUC19-600 was also produced, as detected by primer 4592 (Fig. 4, lane 2). This amount was comparable to the amount of *cspA* transcript from pΔ24T (Fig. 4, compare lane 2 to lane 4; the difference in mobility is due to the 22-base deletion [from base +3 to base +24] of the *cspA* mRNA in the transcript from pΔ24T). This result was again consistent with the high production of CspA in the cells harboring pUC19-600 3 h after cold shock (Fig. 2, lane 2).

The above results demonstrate that the amount of *cspA* mRNA from the chromosomal *cspA* gene increased in the cells overproducing the intact 5′ UTR of *cspA* mRNA 3 h after cold shock. Therefore, we next examined whether this was caused at the level of transcription or by stabilization of the *cspA* mRNA. For this purpose, *E. coli* CL83 cells harboring pUC19-600 and pΔ24T were tested for the stability of the mRNA from the chromosomal *cspA* gene. Cells grown to mid-log phase at 37°C were shifted to 15°C for 3 h. Total RNA was extracted at different time points (0, 5, 10, 20, and 30 min) after the addition of rifampin (200 μg/ml). CL83 cells were used as a control. Primer extension experiments were carried out with primer 3551. The amount of *cspA* mRNA was quantitated by phosphorimager analysis and plotted as shown in Fig. 5. The half-life of the chromosomal *cspA* mRNA from CL83 cells was calculated to be 5.6 min, while the half-lives of the chromosomal *cspA* mRNAs from cells carrying pUC19-600 and pΔ24T were 14.9 and 15.2 min, respectively, indicating that the cold-box deletion did not affect chromosomal *cspA* mRNA stability. The overproduced 5′ UTR from both pUC19-600 and pΔ24T apparently protected the chromosomal *cspA* mRNA from degradation, stabilizing it approximately threefold. Derepression of the chromosomal *cspA* caused by overproduction of the 5′ UTR of the *cspA* mRNA thus occurs at the level of transcription and not at the level of mRNA stability.

Effects of the deletion of the cold box from the chromosomal *cspA* gene. The results described above indicate that the cold-box region is required for the repression of *cspA* at the level of transcription after the acclimation phase. Since these experiments were carried out with multicopy plasmids, we next tested the *cspA* expression of cells containing a single copy of the *cspA* gene with a 22-base deletion at the cold-box region on the chromosome. For this purpose, the *cspA* gene with a deletion from base +3 to base +24 in the 5′ UTR was inserted at the *lpp-28* gene of strain WB002(Δ*cspA*). The resulting strain, WB003(△CB), produced very little, if any, CspA at 37°C, as shown in Fig. 6A. CspA production was highly induced at 30 min after temperature downshift from 37 to 15°C (Fig. 6B). In the parent cells (JC7623), CspA production was significantly reduced at 7 h and was reduced to a very low basal level at 32 h

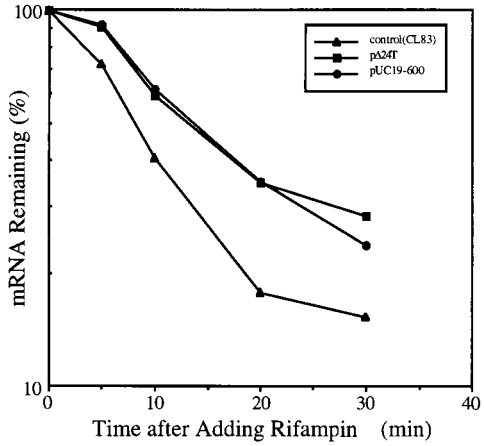

FIG. 5. Stability of chromosomal *cspA* transcript in cells overproducing *cspA* 5′ UTR. CL83 cells harboring pUC19-600 for the 5′ UTR of the wild-type *cspA* gene and pΔ24T for the cold-box-deleted 5′ UTR of *cspA* were grown to mid-log phase and then shifted to 15°C for 3 h. Rifampin (200 μg/ml) was added to the culture, and RNA was extracted at 0, 5, 10, 20, and 30 min after the addition of rifampin. Primer extension was carried out, and the densities of products were analyzed by a phosphorimager and plotted. The amounts of transcripts at the 0 time point were taken as 100.

after temperature downshift. In contrast, a high level of CspA production in WB003 cells was maintained not only at 7 but also at 32 h after cold shock (Fig. 6B), demonstrating that the cold-box region from base +3 to base +24 has a negative effect on *cspA* expression at low temperature.

DISCUSSION

Similar to heat shock response, *E. coli* cells respond to temperature downshift by inducing a specific group of genes, called cold shock genes. Cell growth normally ceases during the first 2 h after temperature downshift from 37 to 15°C, and this period is called the acclimation phase. Interestingly, the synthesis of most cellular proteins is blocked during this phase, except for that of cold shock proteins. Clearly, there is a mechanism for the cold shock proteins to bypass the blockage of general protein synthesis during the acclimation phase. Some of the cold shock proteins synthesized during this phase are ribosome associated and are considered to convert nonfunctional ribosomes to functional ribosomes at low temperature (11, 15).

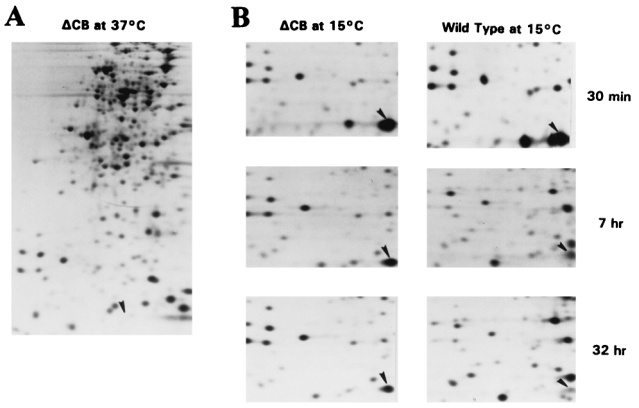

FIG. 6. Constitutive high expression of *cspA* at 15°C resulted from the deletion of the cold-box region from the chromosomal *cspA* gene. Strain WB003 (ΔCB), containing the *cspA* gene with a 22-base deletion from bases +3 to +24 in the 5′ UTR, and its parent strain (wild type), JC7623, were used for labeling cellular proteins. Labeling was carried out at 0.5, 7, and 32 h after a temperature downshift from 37 to 15°C, as previously described (8), and labeled proteins were analyzed by two-dimensional gel electrophoresis. The entire gel is shown only for strain WB003 at 37°C (A). Cell cultures were diluted before they reached the stationary phase, and only the lower parts are shown for 0.5-, 7-, and 32-h time points at 15°C. The positions of CspA are indicated by arrowheads.

The synthesis of the cold shock proteins during the acclimation phase is transient. At the end of the acclimation phase, cells regain the ability to grow, and cold shock protein synthesis is reduced to a new basal level (7). Unlike the heat shock response, no specific transcription factors for cold shock gene induction, such as sigma factors, have been identified. Instead, mRNA stability has been shown to play a major role in the induction of *cspA* (2, 4, 6). We have also unambiguously demonstrated that although the *cspA* gene is constitutively transcribed at 37°C, *cspA* mRNA is designed to be extremely unstable at 37°C (4). Upon cold shock, *cspA* mRNA is immediately stabilized to be ready for protein synthesis, which is consistent with the fact that CspA is instantly produced after temperature downshift (7). Thus, *cspA* induction at low temperature does not require its own specific transcription factor. The *cspA* promoter is very effective at low temperature (4), and as the transcription of *cspA* continues at low temperature, the amount of *cspA* mRNA increases, resulting in higher production of CspA. However, in the middle of the acclimation phase, both CspA protein and *cspA* mRNA production reach their highest levels. After that point, the production gradually drops and is reduced to a low base level at the end of the acclimation phase. It seems evident that there is a mechanism for the repression of *cspA* during the latter half of the acclimation phase.

Here we demonstrate that overexpression of not only the 5′ UTR of *cspA* mRNA (9), but also the 5′ UTR of *cspB*, and *csdA* mRNAs, causes the derepression of all of the cold shock genes. Highly conserved regions consisting of 11 bases were identified within the 5′ UTRs of *cspA*, *cspB*, and *csdA* and designated the cold box (9).

We demonstrated that the deletion of the 22-base cold-box region from the *cspA* 5′ UTR abolished the derepression effect of the 5′ UTR while the overproduction of an mRNA containing the cold-box region was capable of derepression of *cspA* (Fig. 3). The derepression of the chromosomal *cspA* gene was detected not only at the level of CspA production but also at the level of mRNA (Fig. 4). Since the stability of the chromosomal mRNA did not change whether the overproduced 5′ UTR contained the cold-box region or not, the derepression caused by the 5′ UTR containing the cold box occurs at the level of transcription. Since the overproduction of the *cspA* cold-box region causes the derepression of not only *cspA* but also *cspB*, *cspG*, and *csdA*, all of which contain the cold box, it is reasonable to speculate that the cold-box region functions as the binding site of a repressor, as proposed previously (9). The binding of the repressor to the cold box on mRNAs is likely to block transcription.

The proposed function of the cold box is further confirmed by the fact that deletion of the cold-box region from *cspA* on the chromosome also caused derepression of *cspA* expression, resulting in high constitutive expression of *cspA* in cells exponentially growing at 15°C. As expected, the effect of the deletion works only in *cis*, without affecting other cold shock genes, such as *cspB* and *cspG* (Fig. 6B). It should be noted that in addition to *cspA* regulation at the level of transcription, reduction of *cspA* expression after the acclimation phase is partially caused by mRNA destabilization, as shown previously (6). The half-lives of *cspA* mRNAs in CL83 cells changed from 17 min

at 30 min after cold shock to 7.5 min at 1.5 h (data not shown) and 5.6 min at 3 h after cold shock (Fig. 5). What is responsible for the stability changes of *cspA* mRNA after cold shock and how ribonucleases, such as RNase E, RNase K, RNase II, RNase III, and PNPase (1) are involved in the regulation of cold shock gene expression remain to be determined.

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