

Extended -10 Motif Is Critical for Activity of the *cspA* Promoter but Does Not Contribute to Low-Temperature Transcription

Sangita Phadtare^{1*} and Konstantin Severinov²

Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854,¹ and Waksman Institute, Rutgers, The State University of New Jersey, 190 Frelinghuysen Road, Piscataway, New Jersey 08854²

Received 2 May 2005/Accepted 20 June 2005

Bacterial promoters belonging to the extended -10 class contain a conserved TGn motif upstream of the -10 promoter consensus element. Open promoter complexes can be formed on some extended -10 *Escherichia coli* promoters at temperatures as low as 6°C, when complexes on most promoters are closed. The promoter of *cspA*, a gene that codes for the major cold shock protein CspA of *E. coli*, contains an extended -10 motif. CspA is dramatically induced upon temperature downshift from 37 to 15°C, and its cold shock induction has been attributed to transcription, translation, and mRNA stabilization effects. Here, we show that though the extended -10 motif is critical for high-level expression of *cspA*, it does not contribute to low-temperature expression. In fact, transcription from the wild-type *cspA* promoter is cold sensitive *in vitro* and *in vivo*. Thus, transcription appears to play little or no role in low-temperature induction of *cspA* expression.

Most *Escherichia coli* promoters belong to the $-10/-35$ class, which is characterized by the presence of two 6-bp promoter consensus elements positioned approximately 10 and 35 nucleotides upstream of the transcription initiation start point. Within the context of the RNA polymerase (RNAP) holoenzyme, the σ^{70} subunit conserved regions 4.2 and 2.4 recognize the -35 and the -10 promoter consensus elements, respectively, leading to promoter complex formation and transcription initiation (for reviews, see references 14 and 28). Some promoters lack the -35 promoter consensus element and instead contain a TGn extension upstream of their -10 promoter consensus element. The TGn motif of these so-called extended -10 class promoters is involved in specific interactions with an additional region of σ^{70} , conserved region 3.0 (6, 38). This additional contact is strong enough to make promoter complex formation on extended -10 promoters independent of the σ^{70} region 4.2 interaction with the -35 promoter element (21).

Localized melting (opening) of promoter DNA is necessary for the synthesis of RNA. Promoter opening is temperature dependent, and promoter complexes formed on the $-10/-35$ class promoters close below 15°C (41). In contrast, promoter complexes on the extended -10 *galP1* promoter remain open at temperatures as low as 6°C (1, 4, 5, 13). Although the mechanism of this unusual phenomenon is not fully elucidated, the extended -10 motif was shown to be critical for low-temperature opening of *galP1* (13). However, this motif alone is not sufficient, and some extended -10 promoters are sensitive to low temperature (3, 5). Synthesis of the small regulatory RNA DsrA is under temperature control, and it has been shown that the sequence of the -10 box and the spacer region are the essential elements for the thermal response of this promoter (36).

Upon the temperature downshift, the cells exhibit a cold shock response characterized by a transient arrest of cell growth, severe inhibition of general protein synthesis, and transient induction of *cspA* and three of its homologues, *cspB*, *cspG*, and *cspI* (for reviews, see references 9 and 30). Eventually, cell growth resumes but at a reduced rate. CspA homologues are widespread in bacteria and most *csp* genes' promoters contain the extended -10 motif (Fig. 1). For example, promoters of all of the four cold shock-inducible *csp* genes (*cspA*, *cspB*, *cspG*, and *cspI*) in *E. coli* contain a TGn motif. CspE is cold shock inducible in the absence of CspA, CspB, and CspG, and its promoter also contains TGn motif. However, this motif is not strictly conserved and is missing in promoters for genes coding for cold shock-inducible CspA homologues from, for example, *Caulobacter crescentus* (22), *Lactobacillus bulgaricus* (40), *Salmonella enterica* (19), and *Staphylococcus aureus* (18). Nonetheless, based on the published literature suggesting a link between the TGn motif and the low-temperature opening of certain *E. coli* promoters, it is tempting to speculate that the TGn motif contributes to transcription of *E. coli cspA* at cold shock conditions.

It has been reported that upon temperature downshift, CspA accounts for almost 10% of the total cellular protein (17). This cold shock induction is thought to be accomplished at the level of transcription, translation, and mRNA stability (for reviews, see references 9 and 30). One of the unique features of the *cspA*, *cspB*, *cspG*, and *cspI* genes is the unusually long 5' untranslated region. This region is responsible for both the extreme instability of *csp* mRNA at 37°C (a half-life of ~ 12 s) and for stabilization of mRNA at low temperatures (a half-life of ~ 20 min at 15°C) (26). The *cspA* mRNA also contains a unique sequence located 14 bases downstream of the initiation codon, termed the translation enhancing element (35). This element is also present in CspB, CspG, CspI, and CsdA and is thought to enhance translation initiation at cold shock (26). Experiments involving transcription-translation fusions indicated that the *cspA* promoter, which is highly active at 37°C, also contributes to cold shock induction of *cspA* (11, 26).

* Corresponding author. Mailing address: Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Phone: (732) 235-4116. Fax: (732) 235-4559. E-mail: phadtasa@umdnj.edu.

		-35	-10
<i>E. coli</i>	<i>cspA</i>	TGTTGCATCACCCGCCAATGCC	TGCGTTAATGCACATCA
	<i>cspB</i>	CATTGCTTCATATGTTCAACTAT	TGCGTTAATGATTGCG
	<i>cspI</i>	ATTTGCTTAATCTCAATTAATCA	TGCGTTAATAGCTGCG
<i>B. subtilis</i>	<i>cspB</i>	AGTTGTTTTTCTGAAGATTACT	TGTAGAGTAAAGGTA
	<i>cspC</i>	ATTTGCAGTTATTTCCGATTCCT	TGATAATAAAGAAGA
<i>L. lactis</i>	<i>cspA</i>	ATTTGACTTTCTATCAGAGAGG	TGCTACTATCAAGAG
	<i>cspB</i>	GGTTGACATGTAACCTATATAAT	TGATATTAATAGACGAGT
	<i>cspC</i>	ATTTGACTTTGGTAAGAATAGG	TCTTACTATTAAGAGG
	<i>cspD</i>	GTTTGACATTGAGTATAATTACT	TCTTACTATGAAGAAGT
<i>L. plantarum</i>	<i>cspL</i>	TTTTCAGCGATTA AAAA ACTAGGGATG	TGATATACTTAAGCCA
<i>S. typhimurium</i>	<i>cspB</i>	GCTTGCTATGTTTCAGTTAACCA	TGCGTTAATGGTTGFG
<i>T. maritima</i>	<i>cspB</i>	GTATGACTATTTGGCTTAGAAGAT	TGCTAAAACCTTTAGTA
<i>V. cholerae</i>	<i>cspV</i>	TTTTGTTTTAAGCCCTAATAAATTA	TGCTAAAATTATCTC

FIG. 1. Comparison of the promoter regions of genes encoding CspA homologues. The promoter regions of *E. coli cspA* (43), *cspB* (23), *cspG* (29), and *cspI* (47), *Bacillus subtilis cspB* and *cspC* (12), *Lactobacillus lactis cspA*, *cspB*, *cspC*, and *cspD* (49), *Lactobacillus plantarum cspL* (25), *Salmonella enterica* serovar Typhimurium *cspB* (7), *Thermotoga maritima cspB* (48), and *Vibrio cholerae cspV* (8). The -35 and -10 regions are shown by lines, and extended -10 motifs are boxed.

The cold shock transcription of *cspA* has been a topic of some discussion, the conclusion being that transcription is moderately elevated upon temperature downshift. In vivo data, such as those from primer extension, Northern blot analysis, or DNA microarray analysis, demonstrated a 4- to 5-fold increase in *cspA* transcript under cold shock conditions (31). However, none of these analyses can distinguish if the increase in *cspA* transcript was due to cold shock transcription alone, was a combined effect of transcription and mRNA stabilization upon temperature downshift, or was due solely to mRNA stabilization. In the present study, we studied low-temperature transcription of *cspA* through mutational analysis of the *cspA* promoter and in vitro transcription experiments. We show that the *cspA* promoter-extended -10 motif is critical for transcription at all temperatures and that its contribution to low-temperature transcription is mild. We show that transcription from the *cspA* promoter is strongly inhibited at cold shock conditions both in vivo and in vitro at the level of open promoter complex formation. Thus, the high-level induction of CspA upon cold shock is probably entirely due to dramatic stabilization of its mRNA and its cold-efficient translation machinery.

***cspA* transcription in vivo is affected by disruption of the extended -10 motif.** *E. coli* strain JM83 [F^- *araΔ(lac-proAB) rpsL(Str^r)*] (51) and its *cspA* deletion strain (2) were used in this study. The plasmid pJJGO2, containing entire promoter region, 5' untranslated region, and coding region of *cspA* used in this study has been described previously (16). *E. coli* cells grown overnight in LB medium at 37°C were diluted into fresh LB medium. Cells were grown at 37°C were diluted into exponential phase (optical density at 600 nm of 0.5), and part of the cell culture was harvested and used as control. Aliquots of the cells were transferred to a prechilled LB medium at 15°C and the cells were harvested after 1 h of cold shock. The total RNA was extracted by the hot-phenol method described previously (39). The primer extension, Northern blot analysis, and the deoxyoligonucleotide used for the detection of *cspA* were described previously (32, 37, 50). The products were analyzed on a 6% polyacrylamide gel under denaturing conditions. The radioactive measurements of products were carried out by phosphorimaging and quantitative analysis of the bands. As seen from

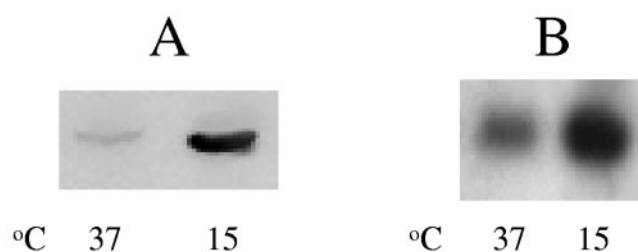


FIG. 2. Changes in *cspA* mRNA levels in response to cold shock. Total RNA was extracted by the hot-phenol method. Primer extension (A) and Northern blot analysis (B) were carried out with deoxyoligonucleotide corresponding to *cspA*. Control (37°C) and cold-shocked (15°C for 1 h) cells were used.

the primer extension (Fig. 2A) and Northern blot (Fig. 2B) analyses, the amount of the *cspA* transcript in wild-type *E. coli* cells is increased ~ 5 -fold upon transfer of exponentially growing *E. coli* culture to 15°C followed by a 60-min incubation at the same temperature. DNA microarray analysis of the cold-shocked *E. coli* also shows a similar increase in the *cspA* transcript level (31, 33).

To investigate the role of the extended -10 motif in *cspA* transcription and cold shock induction, we substituted, by site-directed mutagenesis, the TG of the extended -10 motif by an AC. The mutant promoter, alongside the entire *cspA* transcription unit, was maintained on a plasmid (pJJGO2). A corresponding wild-type *cspA* plasmid was used as a control. Control experiments established that the copy numbers of both plasmids were the same (data not shown). First, we examined if the promoter mutation affects the cold shock induction of CspA. Plasmids carrying wild-type or mutant *cspA* promoters were transformed in *E. coli ΔcspA* cells. Cells were grown at 37°C, and the cultures were split. One half was kept at 37°C, and the other half was subjected to a 1-h cold shock at 15°C. Cell extracts were prepared and subjected to Western blot analysis (32) with anti-CspA antibodies (43). As can be seen from Fig. 3A, CspA was induced by cold shock in cells carrying the wild-type *cspA* promoter, as expected. Disruption of the extended -10 motif strongly affected CspA expression at both 37 and 15°C. At 37°C, the effect was not possible to quantify, since the amount of CspA present in cells carrying the mutant plasmid was beyond the detection limit. At 15°C, the amount of CspA present in cells carrying promoter mutation was increased; however, the final level was >10 -fold less than that in control cells. Thus, it appears that the extended -10 motif makes a critical contribution to the overall strength of the *cspA* promoter but may not participate in cold shock induction.

Transcription from the *cspA* promoter in vitro. To study the possible cold shock-induced transcription from the *cspA* promoter in the absence of RNA degradation that complicates data interpretation, we next studied transcription initiation from the wild-type and the mutant *cspA* promoters in vitro. Promoter complexes were prepared by using *E. coli* RNAP σ^{70} holoenzyme and linear DNA templates extending from position -100 to $+50$ relative to the *cspA* transcription start site at $+1$. Reactions were set up at either 15 or 37°C. Reaction mixtures were supplemented with transcription substrates, the CpA primer, and radioactive ATP, to allow the production of radioactively labeled abortive transcript CpApA. Reaction

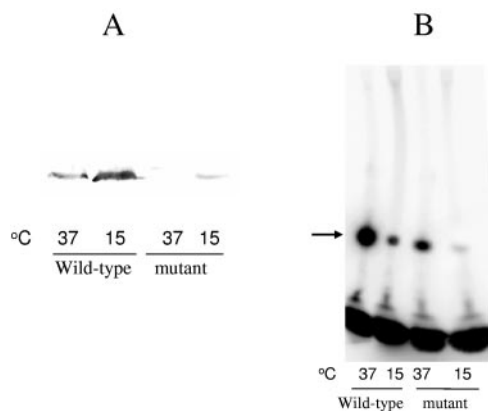


FIG. 3. Effect of disruption of the extended -10 motif on induction of *cspA*. (A) Western blot analysis of *E. coli* $\Delta cspA$ cells carrying pJG02 plasmids with either the wild-type or the mutated *cspA* promoter region was carried out with anti-CspA antibodies. Cell extracts were prepared from cells grown at 37°C and cells cold shocked at 15°C for 1 h. (B) Abortive transcription initiation using DNA templates containing either wild-type or mutated promoter region of *cspA*. The reactions were carried out at 37 or 15°C. Reaction products were separated by denaturing PAGE. The position of the synthesized product (CpApA) is indicated by an arrow.

products were separated by denaturing polyacrylamide gel electrophoresis (PAGE). The results are shown in Fig. 3B. As can be seen, the *cspA* transcription was cold sensitive, and three times less transcript was produced from the wild-type *cspA* promoter at 15°C than that at 37°C. In agreement with the Western blot data, the mutant promoter was less active than the wild-type one at both temperatures. The mutant promoter appeared to be only slightly more cold sensitive than the wild type (five times less transcript produced at 15°C than at 37°C). Identical results were obtained in a runoff transcription assay, indicating that promoter clearance plays no role in cold-resistant transcription from *cspA*.

The experiment presented above assayed abortive initiation from *cspA* at only two temperatures, 15 and 37°C. To better understand the *cspA* response to temperature, we carried out abortive transcription initiation reactions from *cspA* at temperatures ranging from 10 to 37°C. We used, as controls, the *lpp* and *ksgA* promoters. These promoters served as negative controls, since microarray analysis suggested that the RNA synthesized from these promoters is not cold inducible (31). As can be seen from Fig. 4, decreasing reaction temperature led to decreased transcription from all three promoters. Contrary to our expectations, the *cspA* promoter appeared to be the most cold sensitive of the three. Therefore, despite the fact that the steady-state level of the *cspA* transcript increases upon cold shock, low-temperature transcription from the *cspA* promoter is inefficient, at least in vitro from linear DNA.

The *cspA* promoter does not form open complexes at low temperature. In the light of the low-temperature sensitivity of the *cspA* promoter in abortive transcription assays, we examined if this promoter forms open complexes at low temperature. We used KMnO_4 probing (42) to monitor the *cspA* promoter opening at temperatures ranging from 10 to 37°C. The template, which was the same as that used for abortive transcription studies described above, was terminally labeled to

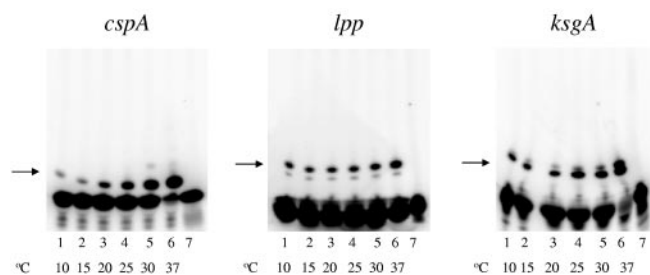


FIG. 4. Temperature-dependent transcription profiles of *cspA*, *lpp*, and *ksgA*. Abortive transcription initiation using DNA templates containing promoter regions of *cspA*, *lpp*, and *ksgA*. The reactions were carried out at indicated temperatures. Reaction products were separated by denaturing PAGE. For *lpp*, CpG and [α - ^{32}P]CTP were used, and for *ksgA*, GpC and [α - ^{32}P]UTP were used. Lane 7 in each case is the respective [α - ^{32}P]NTP loaded on the gel as a control. The positions of the respective synthesized products are indicated by arrows.

visualize probing reaction products. KMnO_4 modifies thymine residues that are present in single-stranded form and thus allows the following of promoter opening. In the absence of RNAP, thymine residues are present in the double-stranded form and are not accessible to KMnO_4 (Fig. 5A, lane 1). The control reactions without RNAP were carried out at both 37 and 15°C and showed the same pattern. At 37°C and in the presence of RNAP, thymine residues at and around the transcription start point became susceptible to the probing agent due to open complex formation (Fig. 5A, lane 7). As the temperature of the reaction was lowered, the intensity of KMnO_4 -sensitive bands uniformly decreased. Little or no KMnO_4 sensitivity was observed below 20°C. Thus, the *cspA* promoter's inability to function at low temperature in vitro is due to the fact that no open complexes form below 20°C.

The experiments carried out above were in a pure system devoid of any cellular factors. We considered a possibility that in vivo, the *cspA* promoter may be aided by some cellular factor(s) that is either induced by cold shock or that may be present even at optimum growth temperature. To explore this possibility, we prepared total cellular extracts from cells that were grown at 37°C and then subjected to a 1-h cold shock at 15°C. As control, cell extracts prepared from cells grown at 37°C were used. The cell extracts were prepared by sonicating the cell pellets followed by centrifugation to clear the cell debris. The extracts were added to abortive transcription initiation reactions from the *cspA* promoter with the idea that a factor(s) present in the extract may allow transcription at low temperature to occur (Fig. 5B). As can be seen from the figure, addition of cell extracts did not lead to increased transcription from *cspA* at low temperature. The origin of faint bands appearing below synthesized product in lanes 3 and 4 is unknown; it may suggest degradation by nucleases present in the cell extract. However, nuclease inhibitors were included in the reactions, and these minor bands did not show variation in response to different amounts of cell extract added. Inclusion of unclarified cell extracts or different amounts of cell extract in abortive transcription assays did not affect the transcription (data not shown). A control reaction (Fig. 5B, lane 5) without *cspA* promoter DNA shows that the cell extract does not contribute to transcription. Results of KMnO_4 probing of pro-

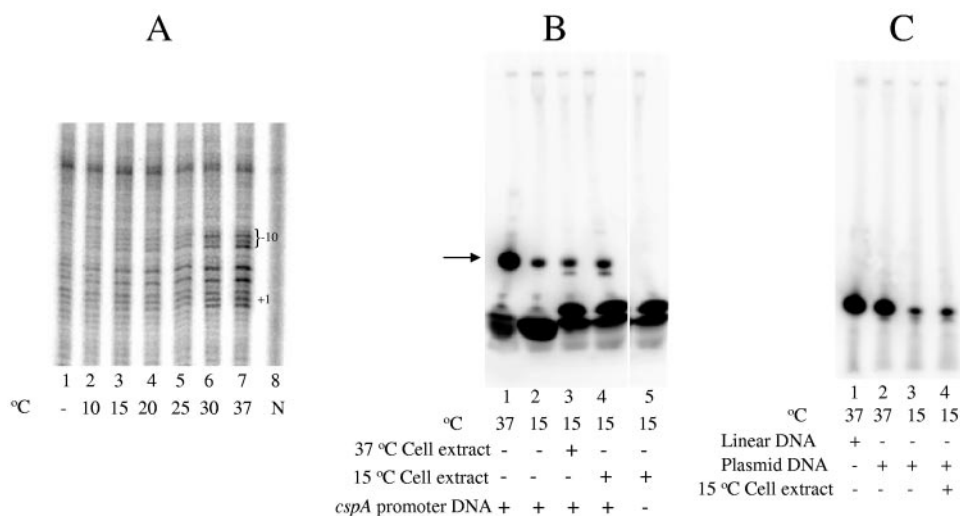


FIG. 5. Promoter opening and transcription of *cspA* at a low temperature. (A) Temperature-dependent open complex formation by the *cspA* promoter. Promoter complexes were formed at indicated temperatures by using *cspA* promoter-containing fragments (both strands were ^{32}P end labeled) and RNAP holoenzyme. Complexes were probed with KMnO_4 and reaction products were separated by urea-PAGE and revealed by autoradiography. Lane 1 represents a reaction without RNAP at 37°C. Lane 8 (N) represents naked DNA without KMnO_4 treatment. Positions of the -10 region and transcription start sites of *cspA* are indicated. (B) Low-temperature transcription of *cspA* in the presence of cell extract. Lanes 1 and 2 represent control reactions carried out at 37 and 15°C, respectively. Lanes 3 and 4 represent reactions carried out at 15°C in the presence of cell extract prepared at 37°C and 15°C, respectively. Lane 5 represents a control reaction carried out at 15°C in the presence of 15°C cell extract, without *cspA* promoter DNA. The position of the synthesized product is indicated by an arrow. (C) Effect of DNA supercoiling on low-temperature transcription of *cspA*. Abortive transcription initiation reactions were carried out by using linear or supercoiled DNA containing the *cspA* promoter. Lane 1 represents a control reaction carried out at 37°C using linear DNA. Lanes 2 through 4 represent reactions carried out by using supercoiled (plasmid-based) DNA containing the *cspA* promoter at the indicated temperatures. Cell extract prepared from cells cold shocked at 15°C for 1 h was included in the reaction represented in lane 4.

motor complexes formed in the presence of cell extracts were consistent with that of transcription assays (data not shown). We also considered the possibility that in vivo, the *cspA* promoter can become open at low temperature in the presence of all four NTPs (such a behavior was reported for a mutant RNAP that forms cold-resistant open complexes [41]). However, even in the presence of high concentration of NTPs, no transcription from and/or promoter opening of the *cspA* promoter was detected (data not shown). Taken together, these data do not support an idea that a specific cellular factor may aid *cspA* transcription during cold shock.

When cells experience cold shock, negative supercoiling of DNA transiently increases, which could favor increased transcription from some promoters (20, 27, 46). To test if the low-temperature sensitivity of the *cspA* promoter depends on DNA topology, we carried out abortive transcription initiation assays using supercoiled plasmid containing the *cspA* promoter (Fig. 5C). In lane 1, transcription products from a control reaction carried out at 37°C by using a linear *cspA* template are shown. Equimolar amounts of supercoiled DNA were used as templates in reactions represented in lanes 2 to 4. At 37°C, *cspA* transcription was not significantly influenced by template supercoiling (compare lanes 1 and 2). Moreover, supercoiling had no stimulatory effect on *cspA* transcription at 15°C (lane 3). The addition of cell extract prepared from cold-shocked cells to reaction mixtures containing supercoiled DNA did not allow low-temperature transcription (lane 4). We therefore conclude that supercoiling of DNA is unlikely to contribute to cold-shock induction of the *cspA* transcript at low temperature.

No open promoter complexes are formed on the *cspA* promoter in vivo. Since our in vitro analysis failed to reveal cold-resistant transcription from the *cspA* promoter, we next followed the *cspA* open promoter complex formation in *E. coli* cells, using in vivo KMnO_4 probing. KMnO_4 probing is a direct measure of open complex formation (and hence, transcription activity of a promoter). Therefore, KMnO_4 probing allows one to avoid the contribution of RNA degradation and/or stabilization that influences the measurements of steady-state levels of mRNA in vivo. The in vivo KMnO_4 probing was carried out as described by Marr and Roberts (24). *E. coli* JM83 cells were transformed with pJG02 plasmids carrying either wild-type or mutant *cspA* promoters. The cells were grown in LB broth at 37°C until exponential phase. Ten milliliters of cells was treated with KMnO_4 . Reactions were terminated by the addition of 11 ml STE (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). Aliquots of 10 ml of cells were also transferred to 15°C and cold shocked for 30 to 60 min and then treated with KMnO_4 . Plasmids were isolated by using the QIAGEN kit. Modified thymine residues were detected by linear PCR (43) with deoxyoligonucleotide used for detection of *cspA* mRNA by primer extension. Equal amounts of plasmid DNA were used in the PCRs. The deoxyoligonucleotide was end labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction products were resolved by using 6% polyacrylamide-urea gels followed by autoradiography. The results are shown in Fig. 6. For each condition, a control reaction without KMnO_4 treatment was carried out to assess the background sensitivity of thymine residues (compare lanes 1, 3, 5, and 7 with lanes 2, 4, 6, and 8, respectively). As expected, the wild-type *cspA* promoter was open at 37°C (lane 1),

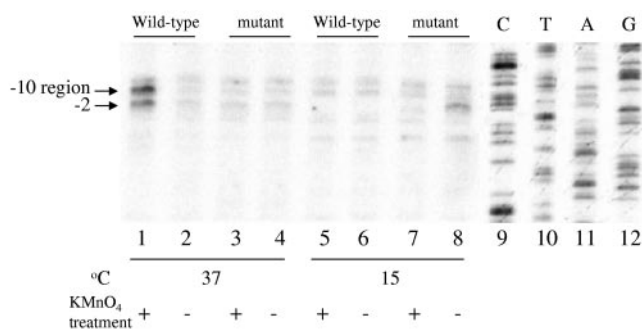


FIG. 6. Temperature-dependent open complex formation on the *cspA* promoter in vivo. In vivo KMnO_4 probing was carried out by using wild-type or mutated *cspA* promoters. Reaction temperatures are indicated. Lanes 2, 4, 6, and 8 represent control reactions carried out without KMnO_4 treatment for lanes 1, 3, 5, and 7, respectively. The KMnO_4 -sensitive bands were positioned with the help of a sequencing ladder shown in lanes 9 through 12. Positions of KMnO_4 -sensitive thymines in the open promoter complex are indicated. The gel is representative of an experiment carried out three times.

while the mutant promoter was closed (lane 3). At 15°C, both promoters were closed (lanes 5 and 7). A control reaction with cold-shocked cells carrying the wild-type *cspA* promoter and treated with rifampin prior to KMnO_4 probing did not reveal any increased sensitivity of the *cspA* promoter thymines (data not shown). Rifampin blocks RNAP escape from the promoter and thus should have increased the level of KMnO_4 sensitivity if RNAP were able to melt the *cspA* promoter in vivo. Based on these results, we conclude that, in agreement with our in vitro analysis, the contribution of the *cspA* transcription to the cold shock induction of *cspA* mRNA is negligible.

Cold shock induction of CspA. The cold shock induction of CspA has been a topic of extensive research over the past few years. The dramatic stabilization of the *cspA* mRNA and its efficient translation at low temperature by virtue of structural elements, such as translation-enhancing element contribute significantly to making CspA a major cold shock protein. On the hand, there has been a dispute regarding the importance of cold shock transcription of *cspA* (15). By studying the expression profiles of reporter genes such as *lacZ* or *cat* fused to the *cspA* promoter, two groups (11, 26) independently showed that the *cspA* promoter responds to temperature downshift (an ~3-fold increase in activity at 15°C). On the other hand, Fang et al. (10) showed that CspA could be induced by cold shock independently of its promoter. However, the cold shock induction of CspA from the heterologous *lpp* promoter was less than that observed from the *cspA* promoter. In vivo data such as primer extension, Northern blot analysis or DNA microarray analysis consistently demonstrate the upshift (4- to 5-fold increase) in *cspA* transcript (31). However, these analyses cannot distinguish if the increase is due to cold shock transcription alone, is a combined effect of transcription and mRNA stabilization upon temperature downshift, or is solely due to message stabilization. Several expression vectors (34, 44, 45) constructed to achieve heterologous expression of proteins at low temperature under transcriptional control of *cspA* also did not include the *cspA* promoter alone but consist of many if not all of the structural elements of *cspA* that contribute to stabilization of its mRNA and cold efficient translation. Thus, it was not

possible to adjudicate the efficiency of cold shock transcription of *cspA* alone.

In the view of the published data that certain promoters containing the extended -10 motif stay open at low temperature, and since this motif is present in several CspA homologues, our aim in the present study was to explore the importance of this motif in cold shock induction of CspA. We initiated the study on the assumption that albeit moderate, *cspA* transcription is elevated upon temperature downshift. Our results showed that TGn motif is essential for efficient transcription of *cspA* at all temperatures and that *cspA* transcription is temperature sensitive. The combined results of the in vitro and in vivo experiments suggest strongly that the contribution of de novo transcription to *cspA* induction at cold shock is marginal. The absence of significant transcription from *cspA* during cold shock is also supported by the following considerations. The steady-state balance between transcription of the *cspA* promoter and the *cspA* mRNA degradation can be described by a simple mathematical formula: $R = (M/t_{1/2}) \times \ln 2$. In this equation, R is the rate of transcription, M is the steady-state level of mRNA, and $t_{1/2}$ is the half-life of mRNA. Considering that the half-life of the *cspA* mRNA increases from 12 s at 37°C to 20 min at 15°C, as little as 5% residual transcription at 15°C (compared to transcription at 37°C) will be sufficient to account for the observed fivefold increase in the steady-state *cspA* mRNA level upon cold shock. Thus, the induction of CspA upon cold shock appears to be solely due to dramatic stabilization of its mRNA and its cold-resistant translation.

We thank Anirvan Sengupta for his help in mathematical modeling of the data.

This work was supported in part by the National Institutes of Health RO1 grant 64530 to K.S.

REFERENCES

- Attey, A., T. Belyaeva, N. Savery, J. Hoggett, N. Fujita, A. Ishihama, and S. Busby. 1994. Interactions between the cyclic AMP receptor protein and the alpha subunit of RNA polymerase at the *Escherichia coli* galactose operon P1 promoter. *Nucleic Acids Res.* **22**:4375-4380.
- Bae, W., P. G. Jones, and M. Inouye. 1997. CspA, the major cold shock protein of *Escherichia coli*, negatively regulates its own gene expression. *J. Bacteriol.* **179**:7081-7088.
- Belyaeva, T., L. Griffiths, S. Minchin, J. Cole, and S. Busby. 1993. The *Escherichia coli* *cysG* promoter belongs to the "extended -10" class of bacterial promoters. *Biochem. J.* **296**:851-857.
- Burns, H., and S. Minchin. 1994. Thermal energy requirement for strand separation during transcription initiation: the effect of supercoiling and extended protein-DNA contacts. *Nucleic Acids Res.* **22**:3840-3845.
- Burns, H. D., T. A. Belyaeva, S. J. Busby, and S. D. Minchin. 1996. Temperature-dependence of open-complex formation at two *Escherichia coli* promoters with extended -10 sequences. *Biochem. J.* **317**:305-311.
- Campbell, E. A., O. Muzzin, M. Chlenov, J. L. Sun, C. A. Olson, O. Weinman, M. L. Trester-Zedlitz, and S. A. Darst. 2002. Structure of the bacterial RNA polymerase promoter specificity sigma subunit. *Mol. Cell* **9**:527-539.
- Craig, J. E., D. Boyle, K. P. Francis, and M. P. Gallagher. 1998. Expression of the cold-shock gene *cspB* in *Salmonella typhimurium* occurs below a threshold temperature. *Microbiology* **144**:697-704.
- Datta, P. P., and R. K. Bhadra. 2003. Cold shock response and major cold shock proteins of *Vibrio cholerae*. *Appl. Environ. Microbiol.* **69**:6361-6369.
- Ermolenko, D. N., and G. I. Makhatazde. 2002. Bacterial cold-shock proteins. *Cell. Mol. Life Sci.* **59**:1902-1913.
- Fang, L., W. Jiang, W. Bae, and M. Inouye. 1997. Promoter-independent cold-shock induction of *cspA* and its derepression at 37 degrees C by mRNA stabilization. *Mol. Microbiol.* **23**:355-364.
- Goldenberg, D., I. Azar, A. B. Oppenheim, A. Brandi, C. L. Pon, and C. O. Gualerzi. 1997. Role of *Escherichia coli* *cspA* promoter sequences and adaptation of translational apparatus in the cold shock response. *Mol. Gen. Genet.* **256**:282-290.
- Graumann, P., T. M. Wendrich, M. H. Weber, K. Schroder, and M. A.

- Marahiel.** 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. *Mol. Microbiol.* **25**:741–756.
13. **Grimes, E., S. Busby, and S. Minchin.** 1991. Different thermal energy requirement for open complex formation by *Escherichia coli* RNA polymerase at two related promoters. *Nucleic Acids Res.* **19**:6113–6118.
 14. **Gross, C. A., C. Chan, A. Dombroski, T. Gruber, M. Sharp, J. Tupy, and B. Young.** 1998. The functional and regulatory roles of sigma factors in transcription. *Cold Spring Harbor Symp. Quant. Biol.* **63**:141–155.
 15. **Gualerzi, C. O., A. M. Giuliadori, and C. L. Pon.** 2003. Transcriptional and post-transcriptional control of cold-shock genes. *J. Mol. Biol.* **331**:527–539.
 16. **Jiang, W., L. Fang, and M. Inouye.** 1996. The role of the 5'-end untranslated region of the mRNA for CspA, the major cold-shock protein of *Escherichia coli*, in cold-shock adaptation. *J. Bacteriol.* **178**:4919–4925.
 17. **Jones, P. G., and M. Inouye.** 1994. The cold-shock response—a hot topic. *Mol. Microbiol.* **11**:811–818.
 18. **Katzif, S., D. Danavall, S. Bowers, J. T. Balthazar, and W. M. Shafer.** 2003. The major cold shock gene, *cspA*, is involved in the susceptibility of *Staphylococcus aureus* to an antimicrobial peptide of human cathepsin G. *Infect. Immun.* **71**:4304–4312.
 19. **Kim, B. H., I. S. Bang, S. Y. Lee, S. K. Hong, S. H. Bang, I. S. Lee, and Y. K. Park.** 2001. Expression of *cspH*, encoding the cold shock protein in *Salmonella enterica* serovar Typhimurium UK-1. *J. Bacteriol.* **183**:5580–5588.
 20. **Krispin, O., and R. Allmansberger.** 1995. Changes in DNA super twist as a response of *Bacillus subtilis* towards different kinds of stress. *FEMS Microbiol. Lett.* **134**:129–135.
 21. **Kumar, A., R. A. Malloch, N. Fujita, D. A. Smillie, A. Ishihama, and R. S. Hayward.** 1993. The minus 35-recognition region of *Escherichia coli* sigma 70 is inessential for initiation of transcription at an “extended minus 10” promoter. *J. Mol. Biol.* **232**:406–418.
 22. **Lang, E. A., and M. V. Marques.** 2004. Identification and transcriptional control of *Caulobacter crescentus* genes encoding proteins containing a cold shock domain. *J. Bacteriol.* **186**:5603–5613.
 23. **Lee, S. J., A. Xie, W. Jiang, J. P. Etchegaray, P. G. Jones, and M. Inouye.** 1994. Family of the major cold-shock protein, CspA (CS7.4), of *Escherichia coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. *Mol. Microbiol.* **11**:833–839.
 24. **Marr, M. T., and J. W. Roberts.** 2000. Function of transcription cleavage factors GreA and GreB at a regulatory pause site. *Mol. Cell* **6**:1275–1285.
 25. **Mayo, B., S. Derzelle, M. Fernandez, C. Leonard, T. Ferain, P. Hols, J. E. Suarez, and J. Delcour.** 1997. Cloning and characterization of *cspL* and *cspP*, two cold-inducible genes from *Lactobacillus plantarum*. *J. Bacteriol.* **179**:3039–3042.
 26. **Mitta, M., L. Fang, and M. Inouye.** 1997. Deletion analysis of *cspA* of *Escherichia coli*: requirement of the AT-rich UP element for *cspA* transcription and the downstream box in the coding region for its cold shock induction. *Mol. Microbiol.* **26**:321–335.
 27. **Mizushima, T., K. Kataoka, Y. Ogata, R. Inoue, and K. Sekimizu.** 1997. Increase in negative supercoiling of plasmid DNA in *Escherichia coli* exposed to cold shock. *Mol. Microbiol.* **23**:381–386.
 28. **Murakami, K. S., S. Masuda, E. A. Campbell, O. Muzzin, and S. A. Darst.** 2002. Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. *Science* **296**:1285–1290.
 29. **Nakashima, K., K. Kanamaru, T. Mizuno, and K. Horikoshi.** 1996. A novel member of the *cspA* family of genes that is induced by cold shock in *Escherichia coli*. *J. Bacteriol.* **178**:2994–2997.
 30. **Phadtare, S.** 2004. Recent developments in bacterial cold-shock response. *Curr. Issues Mol. Biol.* **6**:125–136.
 31. **Phadtare, S., and M. Inouye.** 2004. Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-*csp*-deletion strains of *Escherichia coli*. *J. Bacteriol.* **186**:7007–7014.
 32. **Phadtare, S., and M. Inouye.** 2001. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. *J. Bacteriol.* **183**:1205–1214.
 33. **Polissi, A., W. De Laurentis, S. Zangrossi, F. Briani, V. Longhi, G. Pesole, and G. Deho.** 2003. Changes in *Escherichia coli* transcriptome during acclimatization at low temperature. *Res. Microbiol.* **154**:573–580.
 34. **Qing, G., L. C. Ma, A. Khorchid, G. V. Swapna, T. K. Mal, M. M. Takayama, B. Xia, S. Phadtare, H. Ke, T. Acton, G. T. Montelione, M. Ikura, and M. Inouye.** 2004. Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat. Biotechnol.* **22**:877–882.
 35. **Qing, G., B. Xia, and M. Inouye.** 2004. Enhancement of translation initiation by A/T-rich sequences downstream of the initiation codon in *Escherichia coli*. *J. Mol. Microbiol. Biotechnol.* **6**:133–144.
 36. **Repoila, F., and S. Gottesman.** 2003. Temperature sensing by the *dsrA* promoter. *J. Bacteriol.* **185**:6609–6614.
 37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 38. **Sanderson, A., J. E. Mitchell, S. D. Minchin, and S. J. Busby.** 2003. Substitutions in the *Escherichia coli* RNA polymerase sigma70 factor that affect recognition of extended -10 elements at promoters. *FEBS Lett.* **544**:199–205.
 39. **Sarmientos, P., J. E. Sylvester, S. Contente, and M. Cashel.** 1983. Differential stringent control of the tandem *E. coli* ribosomal RNA promoters from the *rnaA* operon expressed *in vivo* in multicopy plasmids. *Cell* **32**:1337–1346.
 40. **Serror, P., R. Dervyn, S. D. Ehrlich, and E. Maguin.** 2003. *csp*-like genes of *Lactobacillus delbrueckii* ssp. *bulgaricus* and their response to cold shock. *FEMS Microbiol. Lett.* **226**:323–330.
 41. **Severinov, K., and S. A. Darst.** 1997. A mutant RNA polymerase that forms unusual open promoter complexes. *Proc. Natl. Acad. Sci. USA* **94**:13481–13486.
 42. **Severinova, E., K. Severinov, and S. A. Darst.** 1998. Inhibition of *Escherichia coli* RNA polymerase by bacteriophage T4 AsiA. *J. Mol. Biol.* **279**:9–18.
 43. **Tanabe, H., J. Goldstein, M. Yang, and M. Inouye.** 1992. Identification of the promoter region of the *Escherichia coli* major cold shock gene, *cspA*. *J. Bacteriol.* **174**:3867–3873.
 44. **Vasina, J. A., and F. Baneyx.** 1997. Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the *Escherichia coli* *cspA* and *tac* promoter systems. *Protein Expr. Purif.* **9**:211–218.
 45. **Vasina, J. A., and F. Baneyx.** 1996. Recombinant protein expression at low temperatures under the transcriptional control of the major *Escherichia coli* cold shock promoter *cspA*. *Appl. Environ. Microbiol.* **62**:1444–1447.
 46. **Wang, J. Y., and M. Syvanen.** 1992. DNA twist as a transcriptional sensor for environmental changes. *Mol. Microbiol.* **6**:1861–1866.
 47. **Wang, N., K. Yamanaka, and M. Inouye.** 1999. CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold shock. *J. Bacteriol.* **181**:1603–1609.
 48. **Welker, C., G. Bohm, H. Schurig, and R. Jaenicke.** 1999. Cloning, overexpression, purification, and physicochemical characterization of a cold shock protein homolog from the hyperthermophilic bacterium *Thermotoga maritima*. *Protein Sci.* **8**:394–403.
 49. **Wouters, J. A., J. W. Sanders, J. Kok, W. M. de Vos, O. P. Kuipers, and T. Abee.** 1998. Clustered organization and transcriptional analysis of a family of five *csp* genes of *Lactococcus lactis* MG1363. *Microbiology* **144**:2885–2893.
 50. **Xia, B., H. Ke, W. Jiang, and M. Inouye.** 2002. The Cold Box stem-loop proximal to the 5'-end of the *Escherichia coli* *cspA* gene stabilizes its mRNA at low temperature. *J. Biol. Chem.* **277**:6005–6011.
 51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.