Chloramphenicol Induces the Transcription of the Major Cold Shock Gene of *Escherichia coli*, *cspA*

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A downshift in temperature or exposure of cells to certain inhibitors of translation has been shown to induce the synthesis of cold shock proteins in *Escherichia coli*. We characterized the induction of the major cold shock protein (CS7.4, the product of the *cspA* gene) of *E. coli* in response to a shift from 37 to 15°C and in response to the addition of chloramphenicol at 15°C. A pulse-labeling assay and primer extension experiments indicated that the cold shock treatment resulted in a transient increase in the level of the *cspA* transcript and a transient induction of CS7.4, while the addition of chloramphenicol resulted in a constitutive increase in the level of *cspA* transcript and constitutive production of CS7.4. The addition of rifamycin immediately following the temperature downshift or along with the addition of chloramphenicol repressed the transcription of *cspA* as well as the induced production of CS7.4. Furthermore, changes in the *cspA* mRNA level were coincident with changes in CS7.4 synthesis. These results indicate that the expression of *cspA* induced by cold shock and chloramphenicol is at the level of transcription but not at the level of translation. Measurement of the half-life revealed that the *cspA* mRNA induced by chloramphenicol was more stable than that induced by cold shock.

When exponentially growing cultures of *Escherichia coli* are transferred from 37 to 10°C, the rates of synthesis of several cold shock proteins increase (8). The major cold shock protein, CS7.4 (F10.6), a 7.4-kDa cytoplasmic protein, is dramatically induced to 13% of total protein synthesized within the first 2 h following a temperature shift from 37 to 15°C (3). This downshift in temperature results in an increase in the mRNA level of *cspA* (encoding CS7.4), suggesting that the cold shock induction of CS7.4 is at the level of transcription (21). Although the regulatory factors required for the increase in *cspA* transcription have not been identified, in vivo footprinting analysis demonstrated that the region from bases −35 to −73 was protected from chemical modification upon temperature downshift (21). Roles of CS7.4 have been implicated in the cold shock induction of *gyrA* (7) and *hns* (9). A cold shock protein (CspB) from *Bacillus subtilis* that has 61% identity with CS7.4 has been found (24). Similar to the transcriptional induction of *cspA*, transcription of the *cspB* gene also increases following a downshift in temperature (24). In addition to that with CspB, CS7.4 shows high sequence similarity with a protein of 66 amino acid residues from *Streptomyces clavuligerus* (SC7.0; 56% identity [1]) and the "cold shock domain" of eukaryotic Y box factors (2, 19, 20, 25, 26).

It has been proposed that ribosomes can act as sensors of the cold shock response in *E. coli* on the basis of the observation that the cold shock response can be induced by a group of antibiotics (C group) which includes chloramphenicol, tetracycline, erythromycin, spiramycin, and fusidic acid (22). Although the common physiological trigger for cold shock induction is not known, both the downshift in temperature and the C group antibiotics cause a decrease in the nucleotide (p)ppGpp level (10, 11, 14). The variation in the (p)ppGpp level in response to the temperature downshift positively affects the induction of the cold shock response and is adaptive for cell growth (6).

In this study, we characterized the induction of CS7.4 by temperature downshift and by addition of chloramphenicol. Our data indicated that the transient induction of CS7.4 by temperature downshift and the constitutive induction by chloramphenicol were both at the level of transcription, indicating that a common mechanism might exist for the induction. The data shown here also indicated that translation regulation was not involved in the CS7.4 induction by either temperature downshift or the addition of chloramphenicol.

MATERIALS AND METHODS

***E. coli* strain and culture medium.** *E. coli* SB221 (*lpp hsdR trpE5 lacY recY recA*/F' *lacZ*^q *lac*^+ *pro*^+) (13) was used for all experiments and was grown in M9-Casamino Acids medium as described previously (23). For pulse-labeling experiments, an amino acid mixture which lacks methionine was used. The final concentration of each amino acid was 50 μg/ml.

Antibiotics. Chloramphenicol (crystalline) was from Sigma and was used at a final concentration of 30 μg/ml. Kanamycin (kanamycin A) monosulfate was from Sigma and was used at a final concentration of 25 μg/ml. Rifamycin SV (sodium salt) was from Sigma and was used at a final concentration of 0.2 μg/ml.

Pulse-labeling experiments. An overnight culture in M9-Casamino Acids medium was diluted 1:30 with M9 containing the amino acid mixture without methionine. The culture (15 ml) was grown at 37°C, and at a density of 60 Klett units (determined by using a blue filter with a Klett-Summerson colorimeter), the culture was transferred to a 15°C incubator. A portion (1 ml) was labeled with Trans [35S]methionine (1,096 Ci/mmol; 60 μCi/ml) for a 5-min period at 37°C and for a 15-min period at various times after the shift to 15°C; this was followed by the addition of 0.5 ml of 5-mg/ml nonradioactive methionine to stop the labeling. The labeled sample was centrifuged, and the cell pellet was washed with 20 mM sodium phosphate buffer (pH 7.0). The cell pellet was
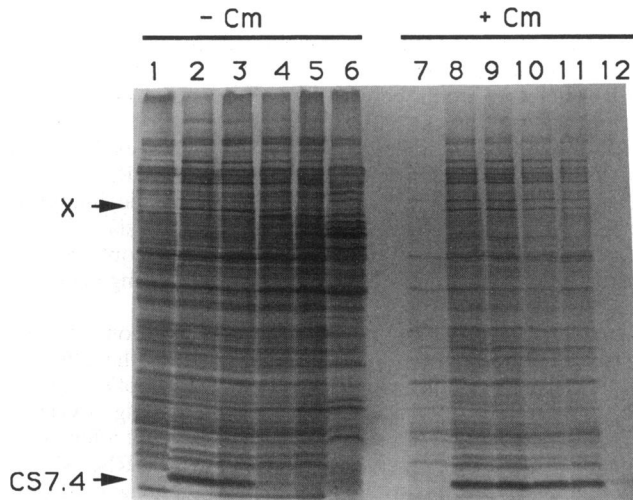
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FIG. 1. Induction of CS7.4 expression by cold shock and by chloramphenicol. The pulse-labeling experiment was done as described in Materials and Methods. Lanes 1 to 6 were for the cold shock response, and lanes 7 to 12 were for chloramphenicol (Cm) (30 μg/ml) induction. For the 37°C sample, chloramphenicol was present for 30 min. For the 15°C samples, chloramphenicol was added immediately after the temperature downshift. Lanes 1 and 7, 37°C; lanes 2 and 8, cold shock for 0.5 h; lanes 3 and 9, cold shock for 1 h; lanes 4 and 10, cold shock for 3 h; lanes 5 and 11, cold shock for 5 h; and lanes 6 and 12, cold shock overnight. X, additional protein induced by cold shock identically to CS7.4.
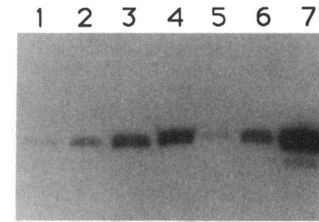

FIG. 2. Regulation of *cspA* expression at the transcriptional level. The amount of the *cspA* mRNA was determined by the primer extension experiment as described in Materials and Methods. Lane 1, 37°C sample without addition of chloramphenicol; lane 2, 37°C sample with chloramphenicol (30 μg/ml) for 30 min; lane 3, cold shock at 15°C for 30 min; lane 4, cold shock for 30 min with chloramphenicol (30 μg/ml); lane 5, cold shock for 5 h; lane 6, cold shock for 5 h followed by chloramphenicol for 30 min; lane 7, cold shock for 5 h with chloramphenicol added immediately after the temperature shift.

resuspended in 40 μl of the same buffer plus 40 μl of 2× sodium dodecyl sulfate (SDS) dye solution. A 20-μl sample was loaded for SDS-polyacrylamide gel electrophoresis using a 17.5% polyacrylamide gel. Electrophoresis was carried out as previously described (4).

Oligonucleotide for primer extension experiment. For the primer extension experiments, a 20-mer oligonucleotide (5′-TAATTAAGTGTGCCTTTCGG-3′) which was complementary to the *cspA* mRNA at the region between the 17th and 36th nucleotides upstream of the initiation codon of *cspA* was used. The primer was labeled at the 5′ end with T4 nucleotide kinase (Bethesda Research Laboratories). For each reaction, 40 pmol of the primer was incubated with a mixture of 80 μCi of [γ-^{32}P]ATP (10 mCi/ml; Amersham), 5 μl of 10× T4 kinase buffer, and 2 μl of T4 kinase (10 U/μl). The final volume was 50 μl. The reaction was done at 37°C for 1 h. The labeled primer was extracted twice with butanol and precipitated with 70% ethanol. The pellet was dried and resolved in 50 μl of 0.1× TE (10 mM Tris HCl, 1 mM EDTA).

Isolation of RNA and primer extension reaction. RNA was isolated as previously described (21). Primer extension reactions using 18 μg of RNA and 2 pmol of the 5′-end-labeled primer were performed as previously described (21).

Other method. ImageQuant software (version 3.0) was used for quick scanning to estimate amounts of the products from the primer extension experiments.

RESULTS AND DISCUSSION

Induction of constitutive *cspA* expression by chloramphenicol during cold shock. A pulse-labeling experiment was carried out to investigate the cold shock response induced by chloramphenicol. In the absence of chloramphenicol, CS7.4

could not be detected at 37°C (Fig. 1, lane 1). After temperature downshift to 15°C, CS7.4 was induced transiently during the first 2 h, which corresponded to the lag time of cell growth caused by cold shock (lanes 2 to 4) (3). However, in the presence of chloramphenicol at 30 μg/ml, CS7.4 was synthesized constitutively at 15°C (Fig. 1, lanes 8 to 12). Even after 24 h, the only detectable band on the protein gel was CS7.4 (lane 12). As expected, the synthesis of most cellular proteins was inhibited to some extent by chloramphenicol. Although it was previously shown that chloramphenicol can also induce *cspA* expression at 37°C (22), we failed to detect the production of CS7.4 at 37°C by chloramphenicol (Fig. 1, lane 7); this was possibly due to the higher concentration of chloramphenicol (30 μg/ml) used in the present experiment. As a control, kanamycin, an antibiotic that induces the heat shock response but not the cold shock response (22), was used in the same experiment, and the results showed no induction of *cspA* expression (data not shown). We also found that the addition of chloramphenicol at 5 h after the temperature shift resulted in constitutive production of CS7.4, indicating that chloramphenicol can induce CS7.4 synthesis de novo at 15°C (data not shown).

Induction of *cspA* expression by chloramphenicol at mRNA level. It has been shown that a downshift in temperature results in an increased level of the *cspA* transcript (21). Thus, a primer extension experiment was performed to determine if chloramphenicol also results in an increased level of the *cspA* mRNA.

At 37°C, almost no *cspA* mRNA was detected (Fig. 2, lane 1). When chloramphenicol (30 μg/ml) was added at 37°C and left for 30 min, the amount of the *cspA* mRNA was slightly increased (Fig. 2, lane 2) even though we could not detect the CS7.4 production on the protein gel (Fig. 1, lane 7). The production of *cspA* transcripts was dramatically induced at 30 min after the cold shock treatment at 15°C (Fig. 2, lane 3). The addition of chloramphenicol immediately after temperature downshift resulted in a slight increase in the amount of the *cspA* mRNA (Fig. 2, lane 4) compared with that induced by temperature downshift (lane 3). In the absence of chloramphenicol, the level of the transcripts decreased to the basal level 5 h after the cold shock treatment (lane 5). However, when chloramphenicol was added at this time point, the mRNA level dramatically increased (30-min incubation; Fig. 2, lane 6). Further incubation in the presence of chloramphenicol (5 h at 15°C) resulted in the accumulation of a large amount of the *cspA* mRNA (lane 7). These results
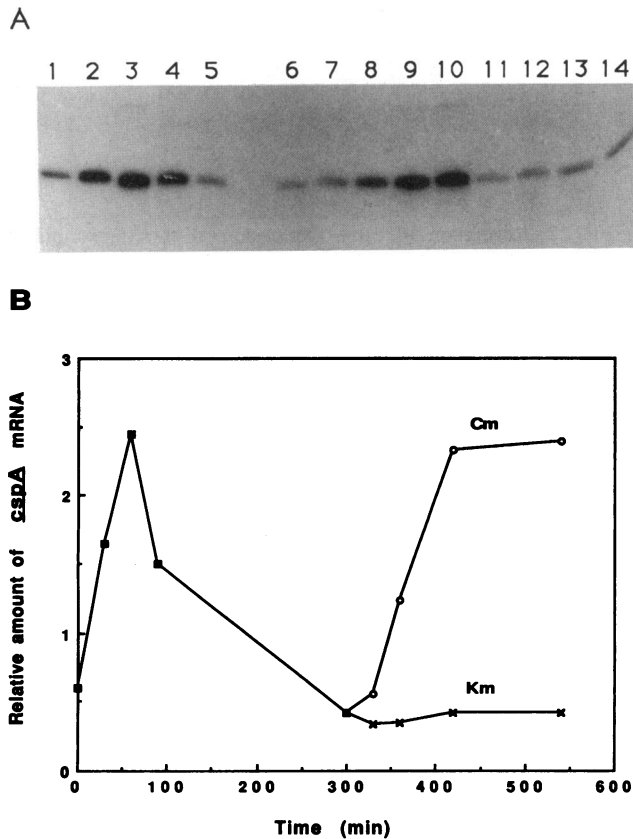
A

B

FIG. 3. Level of *cspA* mRNA produced by the cold shock and chloramphenicol treatments. (A) Primer extension experiments were carried out as described in Materials and Methods. Lane 1, 37°C; lane 2, cold shock at 15°C for 0.5 h; lane 3, cold shock for 1 h; lane 4, cold shock for 1.5 h; lanes 5 and 6, cold shock for 5 h. Lanes 7 to 10 are for the experiments in the presence of chloramphenicol. The drug was added 5 h after the cold shock treatment at 15°C, and the cultures were further incubated for 30 min (lane 7), 1 h (lane 8), 2 h (lane 9), and 4 h (lane 10). Lanes 11 to 14 are the same experiments as in lanes 7 to 10, respectively, except that kanamycin (25 µg/ml) was used instead of chloramphenicol. (B) Amounts of the *cspA* mRNA from panel A as quantitated by a phosphoimager. ■, cold shock; O, chloramphenicol treatment; ×, kanamycin treatment.

clearly demonstrate that chloramphenicol by itself is able to stimulate *cspA* transcription, resulting in the accumulation of the *cspA* transcript. It should be noted that unlike the addition of chloramphenicol after 5 h of cold shock treatment (Fig. 2, lane 6), the addition of chloramphenicol immediately after the cold shock resulted in only a slight increase in the *cspA* mRNA level (lane 4). This suggests that similar mechanisms exist for the transcription induction caused by the cold shock and that caused by the antibiotic. The slight increase in the mRNA level observed in Fig. 2, lane 4, may be due to increased mRNA stability caused by chloramphenicol (see below).

The time course of the *cspA* mRNA induction was examined. In the absence of chloramphenicol, the level of *cspA* mRNA reached a maximum 1 h after the cold shock treatment (Fig. 3A, lanes 2 and 3) and then decreased to a lower basal level (lanes 4 and 5). The 37°C control sample (lane 1) gave a distinct band, since the sample was slightly cold

shocked by storing the cells on ice before they were harvested by centrifugation. When chloramphenicol was added 5 h after the cold shock treatment, the amount of the *cspA* mRNA clearly increased with time (Fig. 3A, lanes 6 to 10). It is interesting that the rate of CS7.4 production induced by chloramphenicol remained constant (Fig. 1, lanes 8 to 11) in spite of the increasing amounts of the *cspA* mRNA during the time course; this was probably due to the inhibitory effect of chloramphenicol on protein synthesis. The increased level of the *cspA* mRNA was also confirmed by using a different primer located within the coding sequence (data not shown).

In order to determine whether the induction of *cspA* transcription by chloramphenicol is specific, the effect of kanamycin (25 µg/ml), one of the H group antibiotics (22), on the *cspA* mRNA level was tested. As shown in Fig. 3A (lanes 11 to 14), the amount of the *cspA* mRNA was not affected by the addition of kanamycin. The levels of *cspA* mRNA shown in Fig. 3A were quantitated by a phosphoimager and are shown in Fig. 3B. These results clearly indicate that chloramphenicol specifically induces *cspA* expression at the level of transcription, like cold shock treatment.

Effect of chloramphenicol on *cspA* mRNA stability at 15°C. Next we investigated whether the increased level of the *cspA* mRNA caused by chloramphenicol at 15°C was due to constitutive activation of the *cspA* transcription and/or to increased stability of the *cspA* mRNA. For this purpose, the half-life of the *cspA* transcript after temperature downshift with and without the addition of chloramphenicol was estimated by adding rifamycin (0.2 mg/ml) (16) immediately after temperature downshift. The level of the *cspA* mRNA was much less in the presence of rifamycin for 30 min (Fig. 4A, lane 9) than it was without rifamycin (lane 2). When rifamycin was added together with chloramphenicol at 5 h after the temperature shift, the level of the *cspA* mRNA dramatically decreased (Fig. 4A, lane 15), indicating that rifamycin was able to block the *cspA* transcription under the conditions used.

Rifamycin was added 30 min after the cold shock treatment, and the amount of *cspA* mRNA was measured at 15, 30, and 60 min after the addition of rifamycin (Fig. 4A, lanes 3, 4, and 5, respectively). One can observe that the *cspA* mRNA was quickly degraded, and almost no *cspA* mRNA was detected at 1 h after the addition of rifamycin (lane 5). As a control, we also measured the amount of the *cspA* mRNA at 45, 60, and 90 min after the cold shock treatment in the absence of rifamycin (lanes 6, 7, and 8, respectively). These time points correspond to those at which measurements were taken in the presence of rifamycin (lanes 3, 4, and 5, respectively). The level of the mRNA was also quantitated by a phosphoimager, as shown in Fig. 4B. The results demonstrate that the *cspA* mRNA, induced by cold shock, was degraded in the presence of rifamycin with a half-life of approximately 15 min at 15°C.

Subsequently the stability of the *cspA* mRNA induced by chloramphenicol was measured. Five hours after a temperature shift to 15°C, the cell culture was incubated for another hour in the presence of chloramphenicol. The stability of the *cspA* mRNA then was measured by adding rifamycin (Fig. 4A, lanes 11 to 14). The rate of degradation of the *cspA* mRNA induced by chloramphenicol became much slower than that induced by temperature downshift (Fig. 4A, lanes 2 to 5). The half-life of the *cspA* mRNA determined by a phosphoimager was approximately 35 min, indicating that the stability of the *cspA* mRNA was increased by chloramphenicol. Thus, the accumulation of the *cspA* mRNA in the
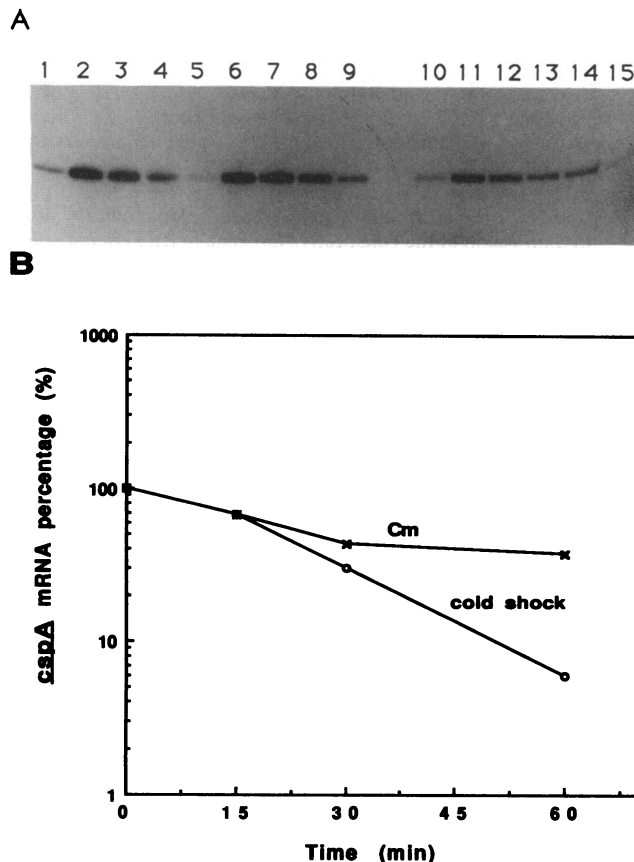
A

B

Time (min)

FIG. 4. Determination of half-life of the *cspA* mRNA. (A) Primer extension was carried out as described in Materials and Methods. Rifamycin (0.2 mg/ml) was used to inhibit *cspA* transcription. Lane 1, 37°C sample; lane 2, cold shock at 15°C for 0.5 h. Rifamycin was added 30 min after cold shock, and samples were taken 15 min (lane 3), 30 min (lane 4), and 60 min (lane 5) after the addition of rifamycin. Lanes 6, 7, and 8 correspond to lanes 3, 4, and 5, respectively, but in the absence of rifamycin. Lane 9, rifamycin was present for 30 min immediately after cold shock; lane 10, cold shock for 5 h; lane 11, chloramphenicol (30 µg/ml) was added at 5 h after cold shock and was present for 1 h. After the chloramphenicol treatment, rifamycin was added, and samples were taken at 15 min (lane 12), 30 min (lane 13), and 60 min (lane 14) after the addition of rifamycin. Lane 15, rifamycin and chloramphenicol were added at 5 h after cold shock and were present for 1 h. (B) Amounts of the *cspA* mRNA from panel A as quantitated by a phosphoimager. O, cold shock; ×, chloramphenicol treatment.

presence of chloramphenicol was considered to be due to the constitutive induction of *cspA* transcription as well as to the increased stability of the *cspA* mRNA. Note that rifamycin was not able to completely block the *cspA* transcription 30 min after the cold shock treatment (Fig. 4A, lane 9).

No translational regulation in *cspA* induction. Because certain inhibitors of translation induce either the cold shock or heat shock response, it has been proposed that the ribosome may be the sensor for the heat or cold shock response (22). Chloramphenicol inhibits protein synthesis by binding to the 50S ribosome subunit (5, 15). It has been demonstrated that translational regulation is involved in the heat shock response, for the increased amount of sigma-32 after heat shock is due to the increased translation of the preexisting mRNA for sigma-32 (12, 18). Thus, we examined
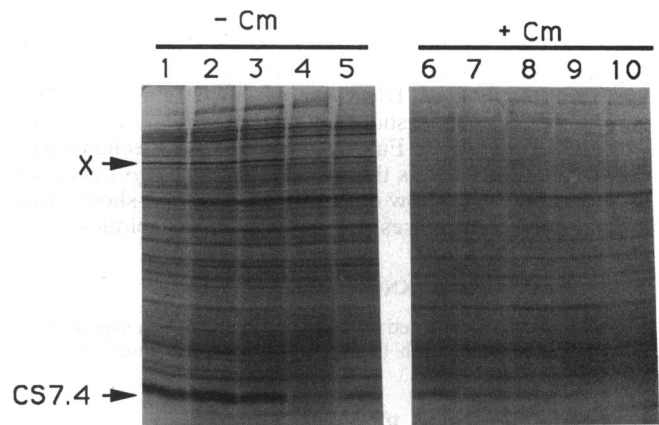
- Cm   **+ Cm**

FIG. 5. Production of CS7.4 during the experiments shown in Fig. 4. Pulse-labeling experiments were carried out as described in Materials and Methods. Lanes 1 to 5 correspond to lanes 2, 3, 4, 5, and 9 in Fig. 4A, respectively. Lanes 6 to 10 correspond to lanes 11 to 15 in Fig. 4A, respectively. X, additional protein induced by cold shock identically to CS7.4. Cm, chloramphenicol.

whether translational regulation is involved in the *cspA* induction by cold shock and chloramphenicol.

For this purpose, the cells were pulse-labeled with [^{35}S]methionine at each time point used for the measurement of *cspA* mRNA stability in Fig. 4A, and the CS7.4 production was compared with the mRNA level. As shown in Fig. 5, lanes 1 to 5, the CS7.4 production induced by the temperature downshift was very similar to the *cspA* mRNA production (Fig. 4A, lanes 2 to 5). Similarly, the CS7.4 production was parallel to the *cspA* mRNA production in the presence of chloramphenicol (compare lanes 6 to 10 in Fig. 5 with lanes 11 to 14 in Fig. 4A). These results suggest that translational regulation is not involved in *cspA* induction by either temperature downshift or chloramphenicol. It is interesting that in both Fig. 1 and 5, in addition to CS7.4 there was another protein (indicated by X) which was induced by cold shock in the identical manner as CS7.4.

Concluding remarks. The results presented in this report demonstrate that the induction of *cspA* expression both by chloramphenicol and by cold shock is at the level of transcription. An important question that needs to be answered is how the *cspA* transcription is induced by chloramphenicol and by temperature downshift. The data presented in this report suggest that a common mechanism exists for the inductions by chloramphenicol and by temperature downshift. It is interesting that the *cspA* expression induced by cold shock is transient and decreases to a new basal level several hours after the cold shock treatment. This significant drop of the *cspA* expression was found to be blocked by the addition of chloramphenicol. This result suggests that in the presence of chloramphenicol, the synthesis of the *cspA* repressor is inhibited and/or the *cspA* activator is constitutively active. Footprinting analysis of the *cspA* promoter region suggests the cold shock induction of a protein factor which binds to the region between bases −35 and −73 upstream of the transcription initiation site (21). Therefore, there may be specific factors which are involved in the induction as well as repression of *cspA*. The (p)ppGpp level, which affects transcription of some *E. coli* genes, decreases in response to chloramphenicol and downshift in temperature (10, 11, 14). The finding that the decrease in (p)ppGpp

positively affects the induction of the cold shock response suggests that variations in the (p)ppGpp level may affect transcription of *cspA* (6). Another regulator of the *cspA* transcription may be DNA supercoiling, which has been implicated in the cold shock regulation of some cold-inducible promoters (7, 17). Further studies of the mechanisms of the induction as well as the repression of the *cspA* gene will provide insight into how cells adapt to the cold shock stress as well as to the stresses caused by some antibiotics.

ACKNOWLEDGMENTS

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