Identification of the Promoter Region of the Escherichia coli Major Cold Shock Gene, cspA

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The major cold shock protein of Escherichia coli, CstA, is produced at a level of 13% of total protein synthesis upon a temperature shift from 37 to 10°C. The transcription of its gene (cspA) was found to be tightly regulated and induced only at low temperature. In addition, the cspA mRNA was extremely unstable at 37°C, so that CstA production was hardly detected when the culture temperature was shifted from 15°C to 37°C. The transcription initiation site (+1) was identified. In vivo footprinting demonstrated that the region from bases -35 to -73 was protected from chemical modification, and gel mobility shift analysis showed that a cold-shocked cell extract contained a factor(s) specifically bound to the fragment containing the sequence between bases -63 and -92. This factor was synthesized de novo only at low temperature, and its synthesis was inhibited by chloramphenicol. Possible functions of this factor are discussed.

When exponentially growing cultures of Escherichia coli are transferred from 37°C to 10 or 15°C, the production of a 7.4-kDa cytoplasmic protein (CstA) is dramatically induced (8). The rate of production of CstA reaches as high as 13% of total protein synthesis within 1 to 1.5 h after the temperature shift to 10°C. The gene for CstA has been cloned; its nucleotide sequence indicated that CstA consists of 70 amino acid residues. Interestingly, CstA shows high sequence similarity with one of the domains of human DNA-binding proteins DbpA, DbpB, and YB-I (6a, 27), raising the intriguing possibility that the conserved sequence in these proteins may play an essential role in gene regulation in both prokaryotes and eukaryotes.

We demonstrated previously that the production of CstA was very tightly regulated, such that CstA synthesis was undetectable at 37°C (8). In this report, we demonstrate that the transcription of the gene for CstA (cspA) dramatically increased upon cold shock. In addition, the cspA mRNA was extremely unstable at high temperature, which also contributes to the exclusive production of CstA at low temperature. A distinct region upstream of the cspA promoter was protected in an in vivo footprinting experiment at low temperature. Subsequently, we were able to identify a factor(s) that bound to a specific region upstream of the cspA promoter. This factor was synthesized de novo only when cells were cold shocked. A possibility that this factor functions as a transcriptional factor for cspA is discussed.

MATERIALS AND METHODS

Bacterial strains and DNA manipulations. E. coli SB221 (Δppk ΔhsdR ΔtrpE5 ΔlacY recA ΔF' lacF+ ΔlacI+Δ pro') (17) was used as the host in all in vivo experiments.

DNA was manipulated as described by Maniatis et al. (13). Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer by the phosphoramidite method and purified by preparative gel electrophoresis as previously described (10, 24). Oligonucleotide-directed site-specific mutagenesis was performed by the plasmid method as previously described (10).

Isolation of RNA. RNA was isolated by the method of Chomczynski and Sacchi (3) with the following modifications. The denaturing solution consisted of 4 M guanidinium thiocyanate (Fluka), 2% sarcosyl, 50 mM Tris-HCl (pH 7.5), 12 mM EDTA, and 0.15 M 2-mercaptoethanol, and all solutions were treated with diethyl pyrocarbonate (Sigma) before they were autoclaved. A 50-ml cell culture was grown in L broth (15) at 37°C until the culture reached a density of 80 Klett units (determined by using a red filter with a Klett-Summerson colorimeter) and then transferred to 15°C.

Before the shift in temperature, a 5-ml aliquot was removed from the culture and centrifuged at 10,000 × g. The pellet was then suspended in 200 μl of the denaturing solution in an Eppendorf tube; 20 μl of 2 M sodium acetate (pH 4) and then 200 μl of phenol and 40 μl of chloroform were added. The samples were extensively vortexed after each reagent was added. All of these steps were done either in a 37°C warm room for the 37°C sample or in a 4°C cold room for the 15°C samples. The samples were placed on ice for 15 min and then centrifuged at 4°C for 15 min, and the top aqueous layer was transferred to a new tube containing 1 ml of 100% ethanol and kept at −20°C for 1 h. After centrifugation, the pellet was resuspended in 60 μl of the denaturing solution and precipitated at −20°C with 200 μl of ethanol for another hour. The resulting pellet was then washed with 200 μl of 75% ethanol and suspended in 200 μl of 1 mM EDTA (pH 8). The samples were then stored at −80°C in 2.5 volumes of 100% ethanol.

Primer extension and RNA sequencing. Primer extension and RNA sequencing were done by procedure 2 of Geliebter et al. (6). Samples of 3 × 10⁴ cpm of 3²P-labeled oligonucleotide primer (Amersham) were used for primer extension, and samples of 3 × 10⁴ cpm were used for RNA sequencing. The primer used for the experiment shown in Fig. 2 was an oligonucleotide that corresponded to the opposite strand of nucleotides 531 through 550 on Fig. 1. Another primer, corresponding to the opposite strand of nucleotides 391 through 410, yielded no start sites of transcription (data not shown). As described above, 12.5% of the RNA preparation

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for each sample was used. The avian myeloblastosis virus reverse transcriptase reactions were carried out at 37°C for 90 min with no actinomycin D. Deoxyribonucleoside triphosphates, which were added to the reaction mixtures at a concentration of 0.15 mM for sequencing, were excluded for the primer extension reactions. Samples of 3 µl were loaded onto an 8% urea sequencing gel.

Functional mRNA half-life determination. The functional half-life of the cspA transcript was determined as described by Pederson et al. (19). Briefly, cell cultures were grown in M9 minimal medium supplemented as previously described (25) to a density of 2 X 10^8 cells per ml at 37°C. The cell cultures were then shifted to 15°C for 30 min, at which time CS7.4 synthesis begins to reach a maximum (8). Rifampin was then added to a final concentration of 0.2 mM/ml to halt RNA synthesis. Half of the culture was immediately shifted back to 37°C, and 1-ml samples of the 15 and 37°C cells were labeled with 20 µCi of Trans35S-Label (ICN) for 5 min at various time points as described below. Protein synthesis was then terminated with 5% ice-cold trichloroacetic acid, and the proteins were precipitated as previously described (7). Immune precipitation was carried out as previously described (23) with rabbit anti-CS7.4 antiserum and rabbit anti-hJLpA antiserum. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as previously described (11). The concentration of polyacrylamide in the resolving gel was 17.5%.

In vivo footprinting. In vivo footprinting was done as described by Saluz and Jost (21) and Sasse-Dwright and Gralla (22). Briefly, cells were grown to the early log phase at 37°C and then shifted to 14°C for 15 min. The cells were then treated with 0.05% dimethylsulfate for 5 min and stopped by adding cold phosphate-buffered saline in the presence of 2-mercaptoethanol. The cells were centrifuged, and chromosomal DNA was isolated by phenol-chloroform extraction and dissolved in 10 mM sodium phosphate (pH 7.5) - 1 mM EDTA. Genomic DNA was heated to 90°C for 15 min and then treated with 0.1 N NaOH at 90°C for 30 min (14). The DNA was neutralized and precipitated with ethanol. The control sample was not subjected to the 14°C cold shock.

Approximately 1 µg of isolated DNA from each sample was analyzed by using a linear polymerase chain reaction. The primer used was a chemically synthesized 24-mer from the sense strand from nucleotides 302 through 325 (5'-CAAGGCAACCCGGCATTAAATAC-3'; Fig. 1). The DNA was extended by Taq polymerase in the presence of 5 mM MgCl_2 with a primer that was 5' end labeled with [γ-32P]ATP. The samples were denatured at 94°C for 1 min, annealed at 60°C for 2 min, and extended at 72°C for 2 min. The whole process was repeated for 30 cycles. The DNA was then precipitated with 2.5 M ammonium acetate, denatured in sequencing sample solution, and analyzed on an 8% urea sequencing gel.

Gel retardation assay. Gel retardation was carried out as described by Fried and Clothers (5). Reaction mixtures had a total volume of 12 µl. The binding buffer consisted of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 50 mM KCl, 8 mM MgCl_2, 7.5% glycerol, 50 µg of bovine serum albumin per ml, and 150 ng of poly(dI-dC). A specified amount of protein was preincubated in the binding buffer on ice for 30 min and then incubated under the same conditions with 0.5 ng of radioactive DNA (approximately 50,000 cpm) end labeled with [α-32P]CTP and the Klenow fragment of DNA polymerase I (13). The samples were loaded onto a 5 or 8% polyacrylamide gel; the buffer was 10 mM Tris-HCl (pH 7.6) - 1 mM EDTA. The gel was run in the circulating buffer at 100 V for 5 h at 4°C.

Preparation of cell extracts. Cells were grown in M9 minimal medium containing 0.2% Casamino Acids (Difco) at 37°C to the mid-logarithmic phase. The unshocked sample was then cultivated at this stage. The shocked sample was then cultivated to 14°C and incubated overnight. After the cells were harvested, washed with 25 mM Tris-HCl (pH 7.6) - 150 mM NaCl - 5 mM EDTA, and disrupted with two passes through a French press at 10,000 lb/ft^2, the protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM. Care was taken not to subject the controls, unshocked sample to a cold shock during the manipulations described above. The following procedures were done at 4°C for both samples. The samples were centrifuged at 10,000 rpm for 15 min in a Sorvall SA600 rotor, and the supernatant was then centrifuged at 45,000 rpm for 2 h in a Beckman 50Ti rotor. The supernatant was saved as the soluble fraction, which was then subjected to ammonium sulfate precipitations of various percent saturations as indicated below. Proteins that precipitated out were collected by centrifugation and dissolved in 20 mM Tris-HCl (pH 7.6) - 2 mM EDTA to a concentration of approximately 15 mg/ml. The samples were dialyzed for 24 h against 200 volumes of the same buffer, which was changed once during dialysis. The dialysis tubing had a molecular weight cutoff of 1,000. The samples were then centrifuged to remove any insoluble material, dispersed into small aliquots, and stored at −80°C until use.

Protein purification. CS7.4 was purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (12) followed by silver staining (16). The identification of the protein was confirmed by determining the amino-terminal sequence by automated gas phase Edman degradation. The purification method will be described elsewhere (2).

Protein concentration was determined by the method of Bradford (1) with bovine serum albumin as the protein standard.

RESULTS
cspA regulation at the level of transcription. To determine whether cspA expression is regulated at the level of transcription or primer extension experiments were carried out to identify the cspA mRNA from RNA prepared from cell cultures grown at 37 and 15°C. When primer A (complementary to the sequence from nucleotides 531 to 550 in Fig. 1A) was used, an mRNA starting from the A nucleotide at position 458 was clearly detected in the RNA preparation from the 10°C culture (Fig. 2, lanes 2 to 5), whereas no mRNA was detected in the RNA preparation from the 37°C culture (lane 1). The amount of cspA mRNA was still low at 10 min after the temperature shift from 37°C to 15°C (lane 2). The level of mRNA reached a maximum between 45 and 75 min after the cold shock treatment (lanes 3 and 4, respectively) and decreased at 120 min (lane 5). The pattern of the cspA mRNA induction appears to agree well with the pattern of CS7.4 production after cold shock at 15°C as previously shown (8); CS7.4 synthesis steadily increased up to 60 min of incubation at 15°C and then decreased to a low basal level. In addition to the transcription initiation at nucleotide 458 (site a1, Fig. 1A) there is another minor initiation site at nucleotide 457 (site a2). Furthermore, there is another very weak possible initiation site at position 508 (site b, Fig. 1A), as shown in Fig. 2. When primer B (complementary to the sequence from nucleotides 591 to 410; Fig. 1A) was used, no transcript was detected (data not
shown), indicating that the cspA promoter is located between nucleotides 410 and 458.

Functional stability of the cspA mRNA. Next, we examined the stability of the cspA mRNA at 15 and 37°C. A culture grown at 37°C was cold shocked at 15°C for 30 min, and then rifampin (0.2 mg/ml) was added to block transcription. One half of the rifampin-treated culture was shifted back to 37°C, and the remaining half was kept at 15°C. At 15-min intervals, aliquots were taken from each culture to label the cells with [35S]methionine for 5 min at the individual culture temperature. The methionine incorporation was stopped by adding trichloroacetic acid. The trichloroacetic acid precipitate was resolubilized and treated with anti-OmpA and anti-CS7.4 sera. The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). The ompA mRNA was extremely stable at 15°C without any obvious reduction of OmpA production during the 60 min of incubation (Fig. 3, lanes 1 to 5). At 37°C, OmpA production was clearly detected for up to 30 min of incubation (lanes 6 to 9), which is consistent with the previous results showing that the ompA mRNA is stable (18, 26). In contrast, the cspA mRNA appears to be less stable even at 15°C, at which the functional half-life can be estimated to be approximately 30 min (lanes 1 to 5). Most importantly, no CS7.4 production
was observed at 37°C even for the first 5 min at the temperature shift (lane 6), indicating that the cspA mRNA is extremely unstable. Since CS7.4 protein was found to be very stable at both 15 and 37°C (data not shown), lack of protein stability cannot account for the absence of CS7.4 at 37°C in this experiment. However, we cannot rule out the possibility that translation of the cspA mRNA is inhibited at 37°C. In either case, E. coli effectively blocks CS7.4 production at higher temperatures.

Identification of the cold shock enhancer region. From the fact that transcription initiation of cspA mainly starts from the A nucleotide at position 458 (Fig. 2), the −35 and −10 regions of the cspA promoter can be deduced to be at the sequences TTGCAT from nucleotides 421 to 426 and CT TAAT from nucleotides 444 to 449, respectively, assuming that the sigma 70 is used for cspA (Fig. 1A). These sequences are different by a few bases from their corresponding consensus sequences, TTGACA for the −35 region and TATAAT for the −10 region (9). Since cspA expression is regulated at the level of transcription, the cspA promoter probably requires a specific cold shock transcriptional factor. At 37°C the cspA promoter is either nonfunctional without the transcriptional factor induced at low temperature or repressed by a repressor, or perhaps the instability of the cspA transcript is sufficient to cause no detectable CS7.4 production at 37°C.

To distinguish these possibilities, we performed in vivo footprinting with the polymerase chain reaction (21, 22). The region from nucleotides 385 to 423 was clearly protected from chemical modification when DNA from the cold-shocked cells (14°C for 15 min; Fig. 4, lane 1) was compared with that of the cells grown at 37°C (lane 2). This region corresponds to the sequences upstream of the transcription initiation site (+1; site a1 at base 458 in Fig. 1A) from bases −73 to −35. Since no protection from the DNA cleavage reaction of DNA from the 37°C culture (lane 2) was observed over DNA from the cold-shocked cells (lane 1), it is unlikely that a repressor protein binds to a sequence near the cspA promoter to block cspA expression at 37°C.

Next, we attempted to identify the upstream DNA sequence where the cold shock transcriptional factor binds. To identify the DNA sequence, gel retardation analysis (5) was carried out with various DNA fragments generated from the DNA fragment from nucleotides 1 to 600 (Fig. 1B). Note that the XbaI site was created by changing the sequence ATT AAA (nucleotides 600 to 605; Fig. 1A) to TCTAGA by site-specific mutagenesis. Fragments 7 to 9 were chemically synthesized. These fragments were end labeled with Klenow enzyme and used for gel retardation analysis. For gel retardation analysis, cell extracts were prepared from a culture grown at 14°C overnight (cold shocked) and from a 37°C-grown culture (unshocked). These extracts were fractionated with ammonium sulfate. From each ammonium sulfate
fraction, an aliquot was used for gel retardation experiments for fragments 1 to 9 (Fig. 1B).

Figure 5A shows the results obtained with fragment 7 and the 0 to 30% ammonium sulfate saturation fractions from the cold-shocked (lane 1) and unshocked (lane 2) cell extracts. It is evident that, in addition to two retarded upper bands found with both extracts, there is an extra band unique to the cold-shocked extract (arrow in lane 1). With the same cell extract, the cold shock-specific band appeared with fragments 2, 4, and 6 but not with fragments 1, 3, 5, 8, and 9 (data not shown). The cold shock DNA-binding protein was also found in the 30 to 45% ammonium sulfate saturation fraction, but the 0 to 30% fraction had much higher specific activity (ca. 10 times greater than that of the 30 to 45% fraction).

These results demonstrate that cold shock induces a specific DNA-binding protein that binds to a sequence in fragment 7. Fragment 7 contains the sequence from nucleotides 366 to 395 (bases −92 to −63; Fig. 1A), which overlaps by 13 bp at the 3' end with the region protected by in vivo footprinting (Fig. 4). It should be noted that fragment 9, which encompasses nucleotides 385 to 414 (bases −73 to −44), was unable to form a cold shock-specific band. Thus it appears that the cold shock factor requires not only the sequence from nucleotides 385 to 395 (bases −73 to −63) but also a sequence a little further upstream of the sequence for its binding.

It should be noted that when purified CS7,4 was used in the binding reaction, no cold shock-specific bands were identified by gel retardation analysis with fragment 1 or 2 (data not shown).

Characterization of the cold shock factor. The cold shock DNA-binding factor was further characterized by gel retardation analysis with fragment 2 (Fig. 1B). When nonlabeled fragment 2 was added in a 100-fold excess, the cold shock-specific band disappeared (compare lanes 1 and 3 in Fig. 5B). In addition, calf thymus DNA had no effect on the band (lane 5). Thus, the appearance of the cold shock-specific band was sequence specific. Preincubation of the cold shock extract with proteinase K eliminated the appearance of the cold shock-specific band (compare lanes 7 and 8 with lanes 9 and 10). Furthermore, heat treatment of the extract (10 min at 80°C) also inhibited the appearance of the band (lanes 11 and 12). These results indicated that the cold shock factor is a protein.

Next, we examined whether the cold shock DNA-binding factor is synthesized de novo upon cold shock or resulted from modification of a preexisting factor (for example, by phosphorylation or by peptide cleavage). For this purpose, the cell extracts were prepared from cells cold shocked in the absence and the presence of chloramphenicol (30 μg/ml).
FIG. 6. De novo synthesis of the cold shock factor. A cell culture growing at 37°C was shifted to 14°C in the presence (+CM) or absence (−CM) of 30 µg of chloramphenicol per ml. The incubation at the cold temperature was continued for 0.5 h (lanes 1 and 4), 1 h (lanes 2 and 5), and 3 h (lanes 3 and 6), and cells were extracted. A 1-µg protein sample was then used for a gel retardation assay with fragment 7 DNA as a probe (Fig. 1). The arrow indicates the cold shock-specific band. Lane 7 had no protein in the binding reaction. The samples were loaded onto an 8% polyacrylamide gel.

The cold shock-specific band with fragment 7 (Fig. 6) can be detected with the cell extract from the cells cold shocked for 0.5 h (lane 4); the intensity of the band became stronger for longer cold shock treatment (1 and 3 h of incubation for lanes 5 and 6, respectively). In contrast, no cold shock-specific band was observed with the extract from the cells cold shocked in the presence of chloramphenicol (lanes 1 through 3), indicating that the cold shock DNA-binding factor is synthesized de novo during cold shock treatment.

DISCUSSION

The present results clearly demonstrate that the production of the major cold shock protein of E. coli, CS7.4, is tightly regulated at the level of transcription. An in vivo footprinting experiment showed that the region from bases −73 to −35 was protected at 14°C but not at 37°C, indicating that there is a cold shock factor(s) binding to this region upstream of the cspA promoter. It is interesting to note that the cold-shocked cell extract contained a factor(s) that bound to the fragment from bases −92 to −63 (fragment 7 in Fig. 1B). However, this factor was unable to bind the fragment from bases −73 to −44 (fragment 9). Thus, it appears that the cold shock DNA-binding factor requires a contiguous upstream sequence beyond base −73 in fragment 7 for its stable binding, although this upstream sequence was not detected in the in vivo footprinting experiment. It remains to be determined whether the region from bases −43 to −35 protected in vivo was due to a secondary factor and whether the cold shock DNA-binding factor is a transcriptional factor for cspA.

It is interesting to note that CS7.4 shows high sequence similarities to human DNA-binding proteins DbpA, DbpB, and YB-1 (29). DbpA and DbpB were found in human placenta and consist of 412 and 364 amino acid residues, respectively (20). Although their functions are unknown, they bind DNA and their internal sequences of approximately 70 residues have a remarkable similarity to almost the entire sequence of CS7.4 (46% identity and 67% similarity for both proteins). On the other hand, YB-1, isolated from human lymph, has been shown to bind to the CCAAT-containing Y box of HLA class II genes (4). Again, an internal domain of YB-1 shows an extensive sequence similarity to CS7.4 (46% identity and 67% similarity). These facts suggest that these human DNA-binding proteins share a functional domain with CS7.4.

Purified CS7.4 had no antifreeze activities associated with the antifreeze proteins of cold-water-dwelling fish (3a). However, since an extremely large amount (ca. 2.5 × 10⁵ molecules per cell) of CS7.4 is produced upon cold shock, CS7.4 is likely to have a general cellular function. The low production of CS7.4 at 37°C is partly attributed to the extreme instability of the cspA mRNA at 37°C. However, it is evident that there is a specific transcriptional activator(s) for cspA at low temperatures. The present study demonstrated that there is a cold shock-inducible (chloramphenicol-sensitive) factor(s) that binds to a specific region upstream of the cspA promoter. Further purification of this factor and characterization of its possible role in the transcription of cspA are now in progress.

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