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Hsc66, an Hsp70 Homolog in *Escherichia coli*, Is Induced by Cold Shock but Not by Heat Shock

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Hsc66 is the second identified Hsp70 protein in *Escherichia coli*. Mutations in *hscA*, the gene encoding Hsc66, compensate for some phenotypic effects of a mutation in *hns*, a gene encoding the cold-inducible, nucleoid-associated protein H-NS. Expression of *hscA* was not induced upon heat shock but was induced approximately 11-fold 3 h after a shift from 37 to 10°C. Furthermore, *hscA* was induced upon chloramphenicol addition, which induces the synthesis of other cold-inducible genes. Mapping of the transcription initiation site showed that *hscA* was cotranscribed with an upstream *dnaJ*-like gene, *hscB*; thus, *hscB* was also cold inducible. The *hscBA* promoter did not contain a Y-box element found in some cold-inducible promoters. Using two-dimensional electrophoresis, we identified Hsc66 under static 37°C growth conditions and showed that Hsc66 was induced, as well as *hscA*, 3 h after a cold shock. Growth of an *hscA* mutant following cold shock was monitored relative to that of an isogenic wild-type strain. While cold shock adaptation as a function of growth rate was not significantly impaired in an *hscA* mutant, the expression of at least five other proteins was altered in this mutant following cold shock. On the basis of the homology to Hsp70 proteins and the induction following cold shock, we speculate that Hsc66 functions as a cold shock molecular chaperone.

Organisms respond to environmental stress by inducing a class of conserved proteins termed heat shock proteins. One subset of these proteins includes the 70-kDa heat shock proteins (Hsp70 proteins). Hsp70 proteins, including the well-characterized *Escherichia coli* DnaK protein (reviewed in reference 2, 9, 10, and 15), function as molecular chaperones assisting unfolded, misfolded, or aggregated proteins in retaining or attaining a specific conformation. Additionally, Hsp70 proteins are involved in protein translocation across cytoplasmic membranes (reviewed in reference 8) and protein degradation (38). The genes encoding Hsp70 proteins are induced following environmental stresses typified by heat shock but including such diverse shocks as ethanol treatment and virus infection.

Hsc66, the second Hsp70 protein found in *E. coli* (23, 35), was identified by screening for mutations that compensated for a specific *hns* mutation. H-NS is a small, dimeric, nucleoid-associated protein in *E. coli* (reviewed in reference 43) whose expression can increase from approximately 20,000 copies under static 37°C growth (37) to three to four times that amount 3 h following a temperature shift from 37 to 10°C (25); thus, H-NS functions as a stress response protein.

Shifting growing *E. coli* from 37 to 10°C immediately inhibits cellular growth and protein synthesis. To compensate for this stress, a protein subset is induced between 1 and 4 h following the temperature shift; this induction is termed the cold shock response (reviewed in reference 20). The cold shock response is analogous to the well-characterized heat shock response in that a temperature shift results in cellular stress and protein induction compensating for potential stress-related cellular damage.

Hsp70 proteins are a major, well-studied component of the heat shock response. However, expression of Hsp70 proteins or molecular chaperones in general has not been identified in the cold shock response. In this report, we show that the gene encoding Hsc66 was not induced following heat shock but was induced following cold shock or the addition of chloramphenicol, which induces other cold-inducible genes. Also, the expression pattern of other proteins induced upon cold shock was altered in an *E. coli* strain lacking Hsc66. We speculate that Hsc66 acts as a molecular chaperone aiding the cell in recovery from cold shock stress.

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MATERIALS AND METHODS

Bacterial strains, plasmids, phage, culture media, and genetic techniques. The bacterial strains, plasmids, and phage used in this study are listed in Table 1. Bacteria were routinely grown in Luria broth alone or Luria broth with 1.5% Bacto-agar (Difco). M9 medium (31) used in labeling experiments was supplemented with 0.4% dextrose, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 5 μg of thiamine per ml, and 0.05 mg of all amino acids except methionine per ml. Generalized transductions using phage P1 *vir* were carried out as described previously (24).

Isolation of total RNA. Total RNA was isolated essentially as described previously (7). Briefly, 10-ml cultures were centrifuged at 4°C, and the cells were resuspended in 600 μl of 10 mM KCl–5 mM $MgCl_2$–10 mM Tris (pH 7.4). This suspension was immediately diluted with 600 μl of hot lysis buffer (0.4 M NaCl, 40 mM EDTA, 1% beta-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 20 mM Tris; pH 7.4) and 100 μl of buffer-saturated phenol (Gibco-BRL) and incubated in a boiling water bath for 40 s. Cellular debris was removed by centrifugation, and the supernatant was extracted three times with phenol-chloroform (5 prime, 3 prime). Residual DNA was removed by extraction with acid phenol-chloroform (Ambion). The RNA was precipitated with isopropanol, pelleted, dried, and resuspended in water. RNA was quantitated with a GeneQuant spectrophotometer (Pharmacia Biotech). A_{260}/A_{280} ratios typically ranged from 1.7 to 1.8.

RNase protection assays. Plasmids pML7, pML10, pML18, and pML21 (Table 1) were linearized with restriction endonucleases and used as templates to generate labeled antisense RNA probes by using the Maxiscript kit (Ambion) according to the manufacturer's instructions. Full-length antisense probes were purified on 8% denaturing polyacrylamide gels. RNase protection assays were performed with the RPAII kit (Ambion) according to the manufacturer's instructions. Hybridization signals were quantitated with a phosphorimager (model 425F; Molecular Dynamics) and ImageQuant version 3.3 software (Molecular Dynamics). Data are presented as sums above background.

Primer extension. Antisense DNA oligonucleotides were labeled with [γ-^{32}P] ATP (Amersham) by using polynucleotide kinase (New England Biolabs) and purified on 8% denaturing polyacrylamide gels. Labeled oligonucleotide (300,000 cpm) was hybridized to total RNA amounts ranging from 25 to 150 μg in 1× first-strand buffer (Gibco-BRL) containing 10 mM dithiothreitol and 2 mM each

TABLE 1. Bacteria and plasmids

Strain, plasmid, or phage	Description	Source or reference
Strains		
ORN116	*thr-1 leuB thi-1* Δ(*argF-lac*)*U169 malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL fhuA2 supE44 hns-1* Φ(*pilA′-lacZYA-kan*)	23
THK38	*thr-1 leuB thi-1* Δ(*argF-lac*)*U169 malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL fhuA2 supE44 hns*$^+$ linked to *tetR*	23
THK80	*thr leu proA2* Δ(*argF-lac*)*U169 galK his argE rpsL supE mtl xyl recBC sbcB hscA2-cat* (has Tn*10* between *hsd* and *serB*)	23
ML59	Same as THK38, except Φ(*hscA2-cat*)	P1 transduction from THK80
Plasmids and phage		
pGEM3Z	Cloning vector with opposing SP6 and T7 promoters	Promega
pGEM4Z	Cloning vector with opposing T7 and SP6 promoters	Promega
pDP31	pUC118 *uncDC*	34
pJJG02	pUC9 *cspA*	12
pJM41	pBR322 *dnaKJ*	30
pTHK100	pUC19 *hscBA*	23
pML7	pGEM4Z with 240-bp *Sal*I-*Eco*RI fragment from pDP31	This study
pML10	pGEM3Z with 204-bp *Eco*RI-*Pvu*II fragment from pJM41	This study
pML18	pGEM4Z with 110-bp *Nru*I-*Hpa*I fragment from pTHK100	This study
pML21	pGEM4Z with 231-bp fragment PCR amplified from pJJG02	This study
P1	*vir*	Laboratory collection

deoxynucleoside triphosphate (United States Biochemicals). The hybridization mixture was denatured at 95°C for 10 min, cooled to 42°C over 99 min, and then incubated at 42°C for 1 h with 200 U of Superscript II reverse transcriptase (Gibco-BRL). One microliter of RNase T_1-RNase A mixture (Ambion) was added, and the mixture was incubated at room temperature for 15 min. The DNA was precipitated and electrophoresed on a denaturing 8% polyacrylamide gel. Sequencing reactions of pTHK100 (Table 1) primed with the same oligonucleotide used in extension reactions were performed with a Sequenase version 2 DNA sequencing kit (United States Biochemicals) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis. THK38 and ML59 were grown in 10 ml of M9 medium lacking methionine to an A_{600} of 0.6 to 0.9. In vivo labeling-grade [^{35}S]methionine (150 μCi) (Amersham) was added and incorporated for 5 min at 37°C or 30 min at 10°C and then chased with 5 ml of cold methionine (5 mg/ml). The cells were centrifuged, washed twice in ice-cold 1× phosphate-buffered saline, and lysed in 45 μl of osmotic lysis buffer (10 mM Tris, 0.3% SDS; pH 7.4). Nucleic acids were removed with 5 μl of 10× nuclease mix (150 mM $MgCl_2$, 100 mM Tris, 500 μg of RNase A per ml, 1 mg of DNase per ml; pH 7.0) by being incubated on ice for 5 min. Proteins were solubilized with 45 μl of SDS boiling buffer (5% SDS, 10% glycerol, 60 mM Tris; pH 6.8) and incubated in a boiling water bath for 5 min. Protein concentrations were determined with the Pierce bicinchoninic acid kit. Beta-mercaptoethanol was added to the sample at 5% (vol/vol).

Two-dimensional electrophoresis was performed by Kendrick Labs, Inc. (Madison, Wis.), as described previously (32). Labeled whole-cell lysate (100 μg) was focused in 2-mm-diameter tubes, using 2% BDH pH 4 to 8 ampholines, for 9,600 V-h. After equilibration for 10 min in buffer O (10% glycerol, 50 mM dithiothreithol, 2.3% SDS, and 0.0625 M Tris; pH 6.8), focused proteins were separated by SDS-polyacrylamide gel electrophoresis on a 13% acrylamide slab gel and then transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore).

Rabbit polyclonal anti-Hsc66 serum (obtained from L. Vickery) was used to detect Hsc66 in Western blots (immunoblots). The serum was preadsorbed with ML59 (*hscA* mutant) whole-cell extract to eliminate cross-reactivity. Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was used as a secondary antibody. Horseradish peroxidase activity was visualized with ECL (Amersham) detection reagents according to the manufacturer's instructions.

Nucleotide sequence accession number. The transcription initiation site of *hscBA* was mapped, and the nucleotide sequence of *hscA* was submitted to GenBank under accession no. U01827.

RESULTS

***hscA* is not heat inducible.** The predicted Hsc66 amino acid sequence has 62% similarity and 42% identity to the well-characterized *E. coli* Hsp70 protein DnaK (23). *dnaK* expression increases severalfold 8 min following a shift from 30 to 42°C (for a review, see reference 14). Since genes encoding many Hsp70 proteins are induced following heat shock, we examined *hscA* and *dnaK* expression both preceding and following heat shock. RNA probes antisense to both *hscA* and *dnaK* with specific homologous regions of 145 and 204 bp, respectively, were generated. These probes were used to detect *hscA* and *dnaK* mRNAs by RNase protection assays (Fig. 1). *dnaK* mRNA levels increased significantly following heat shock as expected, whereas *hscA* mRNA levels were unchanged. Therefore, *hscA* expression was not heat inducible.

***hscA* is induced approximately 11-fold 3 h following a shift from 37 to 10°C.** The *hscA* gene was identified by isolating a

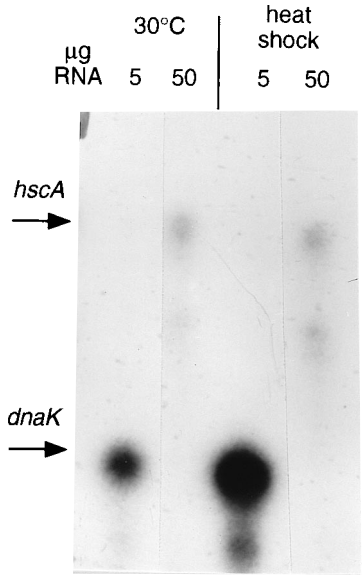

FIG. 1. Results of RNase protection assays depicting *hscA* and *dnaK* mRNA levels following heat shock. Total RNA was isolated from mid-log-phase cultures of ORN116 (Table 1) at 30°C and again following an 8-min temperature shift from 30 to 42°C. RNase protection assays were performed with antisense probes to *dnaK* and *hscA*. The *dnaK* probe was added to only the 5-μg lanes to prevent overexposure in lanes containing greater amounts of RNA.

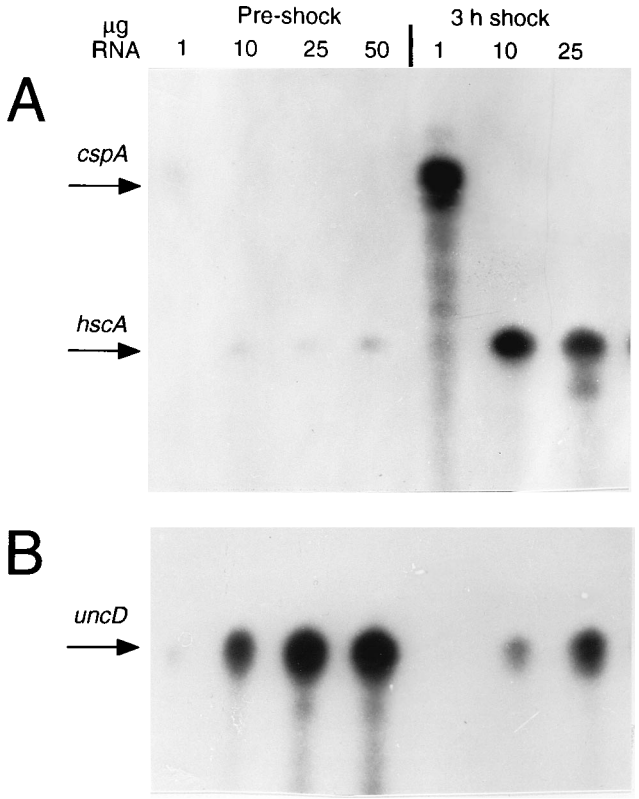

FIG. 2. Results of RNase protection assays depicting *hscA* and *cspA* mRNA levels following cold shock. Total RNA was isolated from mid-log-phase cultures of THK38 (Table 1) at 37°C and again 3 h after a temperature shift from 37 to 10°C. Results from the quantitation of these experiments are presented in Table 2. Different amounts of RNA (as indicated above the lanes) were used with 80,000 cpm of each probe to ensure saturating probe concentrations in at least one lane. More than 10 different RNase protection assays were performed to ensure the reproducibility of these results. (A) Assays with *cspA* and *hscA* antisense probes. The *cspA* probe was added to only the 1-μg lanes to prevent overexposure in lanes containing greater amounts of RNA. (B) Assays with an *uncD* antisense probe. The probe was hybridized separately from the *hscA* and *cspA* antisense probes because of the formation of an anomalous fourth band when all three probes were hybridized to RNA together.

mutation which compensated for the effect of an *hns-1* mutation on *fimA* promoter inversion rates (23). H-NS expression increases following a 3-h cold shock from 37 to 10°C (25). Therefore, we examined *hscA* expression following cold shock. We monitored *hscA* mRNA levels both preceding and 3 h following a shift from 37 to 10°C with an *hscA* antisense RNA probe, using RNase protection assays. As a positive control, we monitored *cspA* expression using an antisense RNA probe specific to 199 bp of *cspA* mRNA in the same manner. The *cspA* gene encodes CspA or CS7.4, the major cold-inducible protein in *E. coli* (12, 22), which is induced severalfold following a shift from 37 to 10°C (41). Both *hscA* and *cspA* mRNA levels increased approximately 11-fold 3 h following a shift from 37 to 10°C (Fig. 2A and Table 2).

An ideal control for these experiments would have been to monitor the mRNA levels of a gene completely unaffected by cold shock. Though expression of some proteins appears to be unaffected by cold shock, no such study at the mRNA level has been performed. Therefore, we measured the mRNA levels of the thermostably expressed gene *uncD* (*atpD*), which encodes the beta subunit of a housekeeping ATPase in *E. coli* (34). *uncD* expression varies little across a variety of static growth temperatures (45). Nevertheless, *uncD* mRNA levels decreased approximately twofold 3 h after a shift from 37 to 10°C (Fig. 2B and Table 2). Although *uncD* expression was affected by cold shock, the fact that *uncD* was repressed following cold shock contrasted with the induction of both *cspA* and *hscA* and thus provided a useful control.

***hscA* is optimally induced 3 h after a shift from 37 to 10°C.** To better characterize the role Hsc66 plays in the cold shock response, we examined the kinetics of *hscA* induction in response to cold shock. *hscA* mRNA levels increased significantly at 2 h, peaked at 3 h, and gradually returned to near-basal levels after 9 h post cold shock (Fig. 3). Furthermore, *hscA* mRNA levels remained above uninduced levels at 5 and 7 h post cold shock (data not shown). CspA protein induction reaches a peak between 60 and 90 min following a shift from 37 to 10°C (12, 41), while *cspA* mRNA levels peak between 45 and 75 min following cold shock (41). The cold shock protein H-NS is optimally induced 3 to 4 h following a cold shift (25). Therefore, the induction time course varies for different members of the cold shock stimulon.

***hscA* is cotranscribed with the upstream *dnaJ*-like *hscB* from a promoter region lacking a Y-box element.** The promoters of *hns* and *gyrA*, another cold-inducible gene, contain multiple Y-box elements (21, 25). CspA is thought to act as a transcriptional activator through interaction with these Y-box elements (3, 21, 25). To determine if *hscA* is regulated by such a mechanism, we first identified the promoter driving its transcription. We used primer extension to map the *hscA* transcription initiation site using nine different antisense DNA probes spanning over 400 nucleotides of both *hscA* and the upstream *dnaJ*-like *hscB* (National Center for Biotechnology Information designation *yfhE*). The two genes, separated by only 19 nucleotides, were cotranscribed (data not shown). We mapped the transcription initiation site of *hscBA* at nucleotide 469 of GenBank accession U01827, corresponding to 57 nucleotides upstream of the *hscB* initiation ATG (Fig. 4). This site was corroborated with primer extension data for three additional oligonucleotides (data not shown). Using these data, we identified a likely promoter between nucleotides 433 and 462. The *hscBA* −10 and −35 sequence regions possessed weak homology to the consensus *E. coli* σ^{70} −10 and −35 sequences (reviewed in reference 13). A potential ribosome binding site (AG AA) was located between nucleotides 518 and 521.

Comparing extension products from RNA samples from cultures grown at 37°C with those from cultures subjected to 3 h of cold shock allowed us to make two additional observations. First, using primer extension as an alternative method to measure mRNA levels, we corroborated the cold shock induction

TABLE 2. Quantitation of *hscA* expression after 3 h of cold shock depicted in Fig. 2

μg of RNA	Shock condition	Pixel vol		
		cspA	*hscA*	*uncD*
1	Preshock	19,857	ND[a]	ND
10	Preshock	ND	11,366	100,083
25	Preshock	ND	13,297	254,048
1	Cold shock	222,431	ND	ND
10	Cold shock	ND	133,098	49,909
25	Cold shock	ND	140,863	105,308
Mean fold change		11	11	−2

[a] ND, not determined.

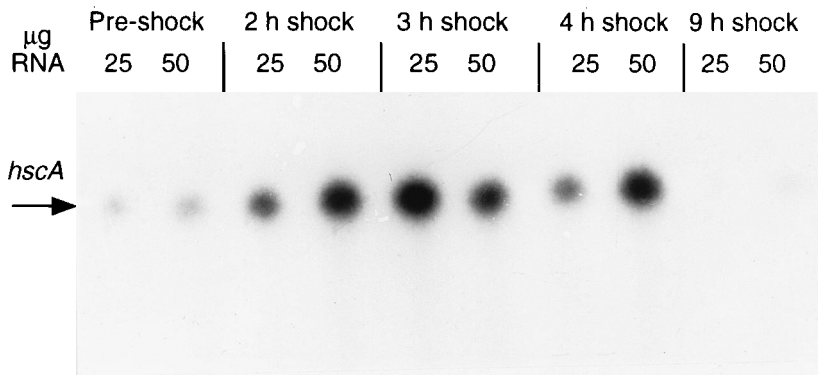

FIG. 3. Results of RNase protection assays depicting *hscA* mRNA levels at various times before and after cold shock. Total RNA was isolated from mid-log-phase cultures of THK38 (Table 1) at 37°C and again at the indicated times following a temperature shift from 37 to 10°C. The indicated amounts of total RNA were hybridized with 80,000 cpm of labeled antisense *hscA* probe to ensure saturating signal concentrations. The decrease in signal seen from the 25- to 50-μg 3-h lanes may be due to the presence of a contaminating RNase.

of *hscA*. Second, extension reactions with an oligonucleotide in *hscB* (Fig. 4A) showed an increase in *hscB* mRNA in cold-shocked samples. Therefore, the *dnaJ*-like *hscB* is cold inducible as well as *hscA*. Although there were some faint additional primer extension products (Fig. 4A, 25-μg cold shock lane), these products were not consistently produced in extension reactions with other oligonucleotides. However, we cannot exclude the possibility that there are weaker *hscBA* transcription initiation sites in addition to the one we have identified.

***hscA* is induced upon chloramphenicol addition.** Previous studies have shown that chloramphenicol addition induces cold shock proteins (18, 44). We grew identical wild-type *E. coli* cultures (THK38) to an A_{600} between 0.4 and 0.5. We isolated total RNA from one culture and added chloramphenicol to a final concentration of 30 μg/ml to the other. This second culture was incubated aerobically at 37°C for 30 min, after which we isolated total RNA. These total RNA samples were used to measure alterations in *hscA* and *cspA* mRNA levels due to the chloramphenicol treatment. Both *hscA* and *cspA* mRNA levels increased following chloramphenicol treatment (Fig. 5); *hscA* expression increased at least 8-fold and *cspA* expression increased approximately 1.5-fold (1-μg lane), as determined by phosphorimaging. Therefore, *hscA* expression, like that of other cold-inducible genes, was induced by the presence of chloramphenicol.

Hsc66, the *hscA* protein product, is cold inducible. Since *hscA* mRNA levels increased following cold shock, we examined Hsc66 levels in cold-shocked versus statically grown cultures. Whole-cell extracts prepared before and after cold shock were labeled and separated via two-dimensional gel electrophoresis and transferred to polyvinylidene difluoride membranes. Western blots probed with polyclonal Hsc66 antiserum were used to locate the labeled spot corresponding to Hsc66 (data not shown). Figure 6 shows Hsc66 expression before and 3 h after cold shock (Fig. 6A and B, respectively). The larger signal in Fig. 6B demonstrates that Hsc66 is cold inducible, as is the *hscA* gene.

An *hscA* insertion mutation does not affect the growth rate but does affect the protein profile of cold-shocked cells. We monitored the A_{600}s of cultures following a 37 to 10°C cold shock of an *hscA* mutant containing the *hscA2-cat* allele (ML59) and an isogenic *hscA*$^+$ strain (THK38). The growth rates of ML59 and THK38 following cold shock were not appreciably different (data not shown). However, THK38 consistently achieved a slightly higher optical density ($A_{600} \approx 1.8$) than ML59 ($A_{600} \approx 1.5$) during stationary phase; thus, the *hscA2-cat* allele does not significantly affect growth rates following cold shock.

To better assess the role of Hsc66 in the cold shock response, we examined the proteins produced in THK38 and ML59 following cold shock. As expected, Hsc66 was absent in ML59 whole-cell lysates, while the expression of at least five other proteins in *hscA2-cat* cells was affected following cold shock (Fig. 6B and C). Thus, the absence of Hsc66 alters the *E. coli* cold shock protein profile.

DISCUSSION

DnaK has long been considered the only Hsp70 protein expressed in *E. coli*. However, we (23) and Seaton and Vickery (35) recently described a second Hsp70 in *E. coli*, Hsc66. Unlike *dnaK* mRNA levels, which increase upon heat shock, *hscA* mRNA levels were unaffected by heat shock. Furthermore, DnaK is repressed upon a 13°C or greater drop in temperature (19), whereas a similar temperature shift results in Hsc66 induction. Therefore, it is unlikely that Hsc66 contributes to the heat shock response, but it may function as a counterpart to DnaK in the cold shock response.

We tested *hscA* cold shock induction because of the genetic link to *hns*. We initially found *hscA* by screening for second-site mutations compensatory to an *hns-1* mutation that affects *fimA* promoter inversion rates. Since *hscA* mutations compensate for only a subset of the *hns-1* mutant phenotypes, Hsc66 may alter the relative proportion of different H-NS conformations, which may possess different biological functions. On the basis of this association with H-NS and the fact that Hsp70 proteins are typically induced under stress conditions, we examined *hscA* expression following cold shock, which induces H-NS expression. CspA is the major cold-inducible protein, and it represents up to 13% of the total cellular protein 90 min after cold shock (12). Because of the predominant role *cspA* plays in the cold shock response, we monitored its expression as a positive control. The increase in both *hscA* and *cspA* mRNA levels (11-fold) following cold shock suggests that *hscA* is a member of the cold shock stimulon.

Jiang et al. previously showed that *cspA* mRNA levels increase fourfold 1 h following cold shock and return to near-basal levels 3 h following cold shock (18). Though we did not perform a time course analysis of *cspA* mRNA levels, our data are inconsistent with this previous study. In our RNase protection assays, *cspA* mRNA levels remained induced 11-fold 3

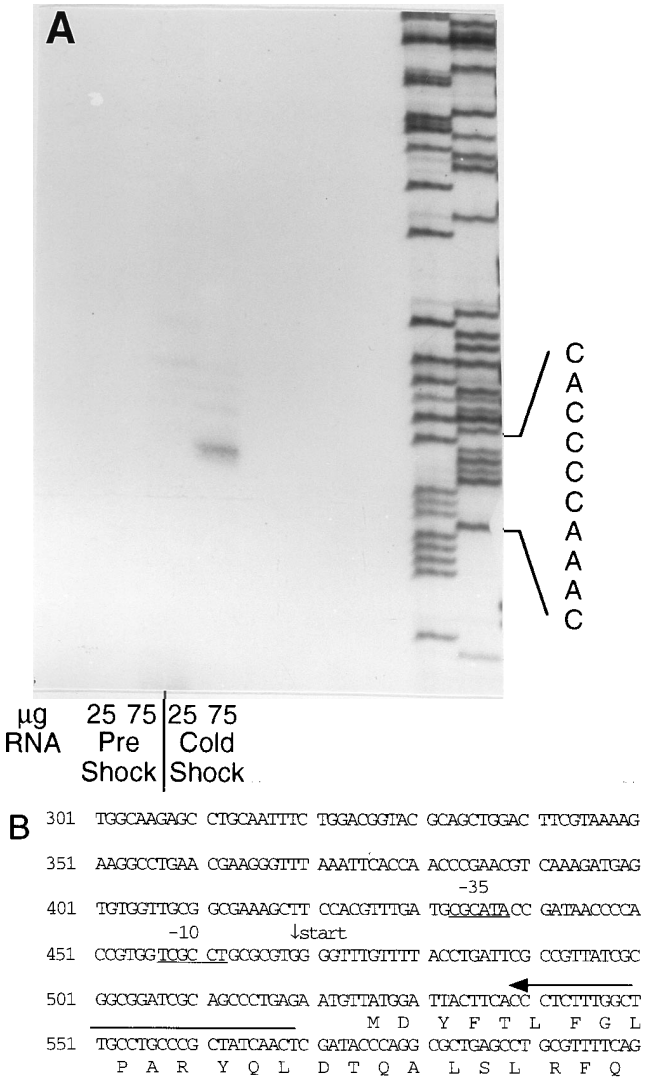

FIG. 4. Mapping the transcriptional start site of *hscA* by primer extension. (A) Total RNA was isolated from mid-log-phase cultures of THK38 at 37°C and again 3 h after a temperature shift from 37 to 10°C. A sequencing reaction was performed with the same oligonucleotide with pTHK100 as a template. (B) Sequence from GenBank accession U01827, with the transcriptional initiation site at nucleotide 469 indicated (arrow). The −10 and −35 regions (underlined) and the sequence corresponding to the oligonucleotide used in the primer extension in panel A (overlined with an arrow) are shown. The initial translation of *hscB* is shown.

h following cold shock. Jiang et al. used primer extensions to quantitate *cspA* mRNA levels, and it is possible that differences in our techniques may account for this discrepancy. Also, at least three other *cspA* homologs exist in *E. coli*: *cspB*, *cspC*, and *cspD* (26). Of these, only *cspB* is cold inducible, in addition to *cspA*. It is possible that our 231-bp antisense *cspA* RNA probe hybridized to *cspB*. If *cspB* mRNA levels remain induced at 3 h following cold shock, in contrast to *cspA* levels, then this might explain the discrepancy.

Though it is clear that *hscA* mRNA levels increased 3 h following cold shock, we wanted to determine at what point after cold shock *hscA* mRNA levels peaked. Expression of *hscA* is optimally induced at 3 h post cold shock. Though we cannot predict the function of Hsc66 on the basis of this finding, it seems unlikely that Hsc66 regulates induction of a subset of the cold shock stimulon like CspA. Immediately upon cold shock, cellular growth and protein synthesis halt. Resumption of growth and protein synthesis at the lower temperature occurs 4 h following the cold shock (22, 36). Because Hsc66 is optimally induced prior to the resumption of sustained growth, it may play a role in enabling the cellular machinery to function properly following a cold shock stress.

Determining at what point in the cold shock response a gene is optimally induced can provide a clue to the function of its product. Products of genes induced early may function as activators. Such is the case with CspA; it is optimally induced 1 h following cold shock and acts as a transcription activator inducing cold shock gene expression (3, 21, 25). H-NS, which is activated by CspA, likely acts as a cold shock transcriptional repressor. Under non-cold shock conditions, H-NS acts as a direct transcriptional repressor of the osmotically induced *proU* operon (42) as well as of numerous other genes (reviewed in reference 43). Optimal cold shock induction of H-NS occurs between 3 and 4 h following the temperature shift (25), later in the cold shock response than optimal induction of CspA. CspA and H-NS induction patterns are consistent with the hypothesis that proteins induced early act as regulatory elements while proteins induced late perform functions directly required by the cold-shocked cell.

To better understand the potential induction mechanism of *hscA*, we wanted to determine the transcription start site and promoter. We used primer extension analysis of mRNAs prepared from cells grown at 37°C and cold-shocked cells to map the *hscA* transcription initiation site used under these two conditions and confirmed that *hscA* mRNA is induced following cold shock. Oligonucleotides designed to anneal to *hscA* sequences were initially used in mapping the transcription initiation site. Accurate mapping of the transcription start site required oligonucleotides hybridizing to upstream *hscB* sequences, proving that *hscB* and *hscA* are cotranscribed. The two RNA sources yielded the same transcription initiation site (data not shown). Therefore, it is likely that the same promoter was used for *hscBA* expression under the two environmental conditions. Additionally, visualizing primer extension products required significantly less cold-shocked RNA than preshocked

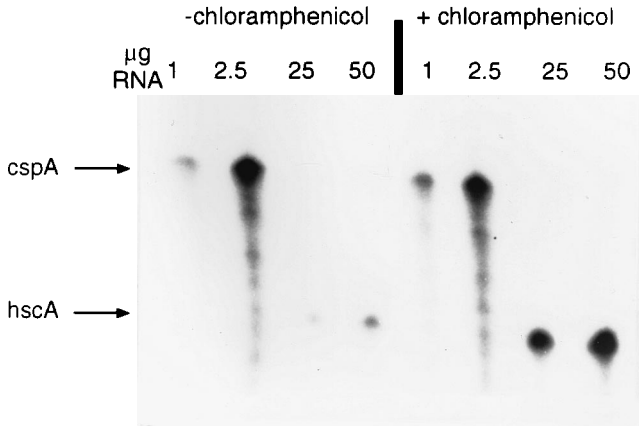

FIG. 5. Results of RNase protection assay depicting *hscA* and *cspA* mRNA levels following chloramphenicol addition. Total RNA was isolated from mid-log-phase cultures of THK38 at 37°C and again 30 min after the addition of chloramphenicol (final concentration, 30 µg/ml). Multiple amounts of RNA were used with 80,000 cpm of each probe to ensure saturating concentrations in at least one lane. *cspA* probe was added to only the 1- and 2.5-µg lanes to prevent overexposure in lanes containing greater amounts of RNA. This experiment was reproduced three times.

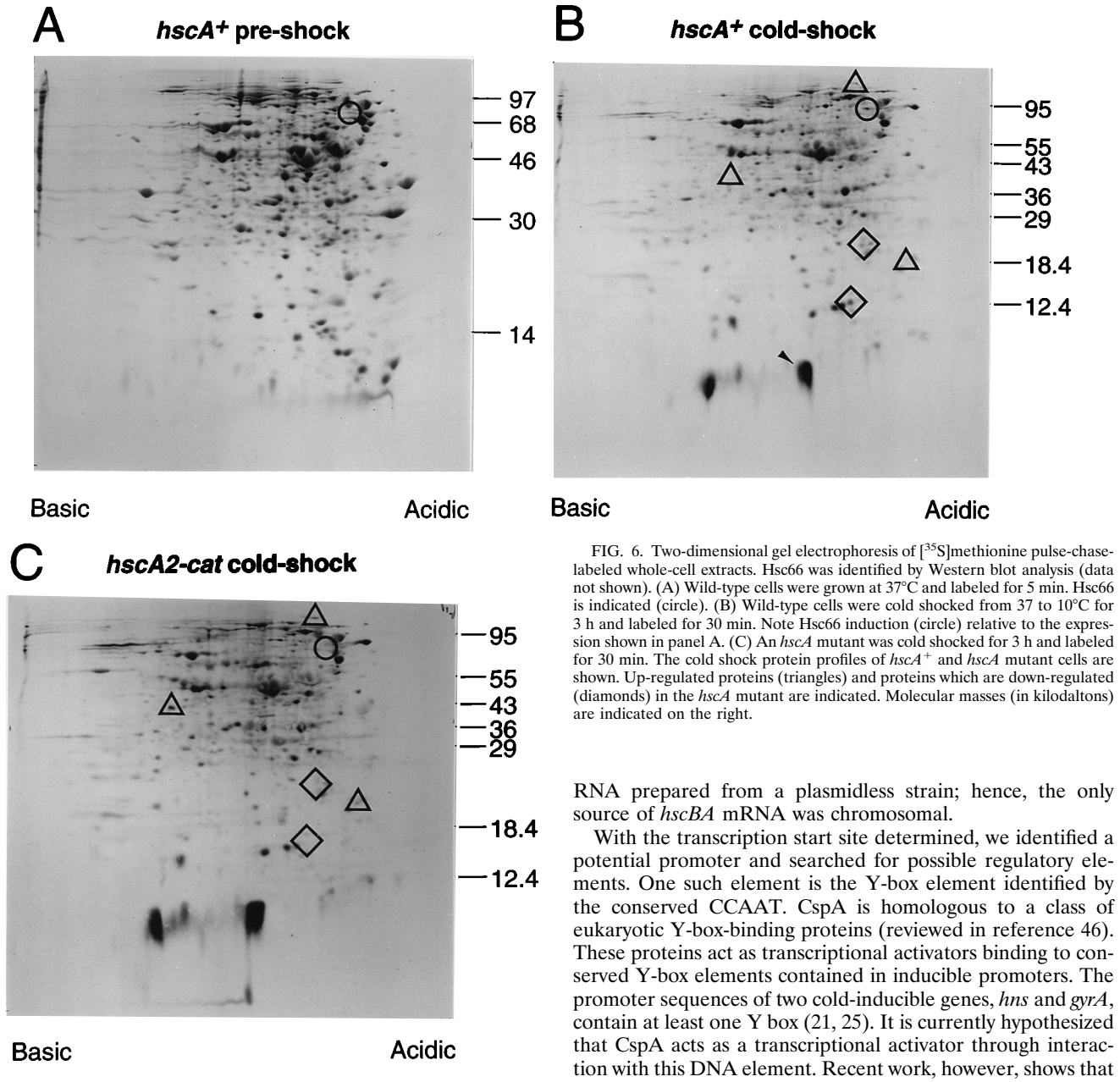

FIG. 6. Two-dimensional gel electrophoresis of [^{35}S]methionine pulse-chase-labeled whole-cell extracts. Hsc66 was identified by Western blot analysis (data not shown). (A) Wild-type cells were grown at 37°C and labeled for 5 min. Hsc66 is indicated (circle). (B) Wild-type cells were cold shocked from 37 to 10°C for 3 h and labeled for 30 min. Note Hsc66 induction (circle) relative to the expression shown in panel A. (C) An *hscA* mutant was cold shocked for 3 h and labeled for 30 min. The cold shock protein profiles of *hscA*$^+$ and *hscA* mutant cells are shown. Up-regulated proteins (triangles) and proteins which are down-regulated (diamonds) in the *hscA* mutant are indicated. Molecular masses (in kilodaltons) are indicated on the right.

RNA, thus corroborating *hscBA* cold induction. It should be noted that a third gene, *fdx*, encoding a [^{2}Fe-^{2}S] ferredoxin lies immediately downstream and is cotranscribed with *hscBA* (39, 40). It is likely that this gene is cold inducible as well, though it is unclear what utility *fdx* induction would provide for cold shock survival, as the function of the *fdx* gene product is unknown.

The transcription initiation site (nucleotide 469 of GenBank sequence U01827) determined in the present study is at odds with the *hscA* transcription initiation site reported previously (35). However, the total RNA used for primer extensions by Seaton and Vickery was extracted from cells containing a cloned copy of *hscA* provided in *trans*. Furthermore, this cloned DNA molecule did not contain the upstream *dnaJ*-like *hscB* gene and did not contain an authentic chromosomal promoter. We determined the transcriptional start site using total RNA prepared from a plasmidless strain; hence, the only source of *hscBA* mRNA was chromosomal.

With the transcription start site determined, we identified a potential promoter and searched for possible regulatory elements. One such element is the Y-box element identified by the conserved CCAAT. CspA is homologous to a class of eukaryotic Y-box-binding proteins (reviewed in reference 46). These proteins act as transcriptional activators binding to conserved Y-box elements contained in inducible promoters. The promoter sequences of two cold-inducible genes, *hns* and *gyrA*, contain at least one Y box (21, 25). It is currently hypothesized that CspA acts as a transcriptional activator through interaction with this DNA element. Recent work, however, shows that binding to the *hns* Y-box element requires RNA polymerase but that transcription activation following cold shock can occur without the Y box (3). The absence of a Y-box element in the *hscBA* promoter region does not prove that *hscBA* cold induction is independent of CspA. We are not aware of any *cspA* mutations that would allow us to test the possible role of CspA on *hscBA* expression. However, we have found that supplying additional copies of *cspA* on a plasmid has no apparent effect on *hscBA* expression (preliminary, unpublished result). This result suggests the possibility that *hscBA* induction is independent of the CspA regulon.

Induction of the entire cold shock stimulon requires at least one additional mechanism other than direct transcriptional activation by CspA binding to a Y-box element. In fact, there is an unidentified cellular component that binds to a 23-bp region of the *cspA* promoter (41). This factor may induce cold shock activators, including CspA, which in turn activate cold-inducible genes such as *hscA*. The *hscA* promoter region lacks

this 23-bp region. Further work on this unidentified cold shock regulator will help illuminate the induction mechanism of the cold shock stimulon.

Though the *hscA* cold shock induction mechanism is not known, we were able to induce *hscA* with chloramphenicol. Adding chloramphenicol to cells growing at 37°C induced *hscA* and *cspA* expression. Thus, both *hscA* and *cspA* are induced by a chemical which produces a potential cold shock signal. Chloramphenicol induces members of the cold shock stimulon, because both the addition of chloramphenicol and a decrease in temperature result in a decrease in GTP-3′-diphosphate and GDP-3′-diphosphate, collectively abbreviated (p)ppGpp (28, 29, 33). Chloramphenicol lowers (p)ppGpp levels by inhibiting peptidyl transferase, leaving a charged tRNA molecule occupying the ribosomal A site. RelA, a ribosome-associated (p)ppGpp synthetase, does not produce (p)ppGpp in the presence of charged tRNAs (reviewed in reference 6). The decrease in (p)ppGpp levels produced by both chloramphenicol addition (28) and a decrease in temperature (29, 33) may act as an inducing signal for the cold shock stimulon (19).

Because (p)ppGpp is produced by the ribosome-associated RelA protein, the ribosome may act as a sensor for cold shock (19, 44). Although this is an exciting possibility, a direct physical link between a decrease in (p)ppGpp concentration and the specific induction of the genes responsible for the cold shock response has yet to be established. It is interesting to speculate that (p)ppGpp may alter RNA polymerase specificity (16) with the assistance of a cold shock activator to induce the cold shock regulon. The fact that chloramphenicol increases *hscA* mRNA levels under non-cold-shocked conditions supports the notion that (p)ppGpp is involved in signaling the induction of the cold shock stimulon.

In addition to studying the *hscA* induction mechanism, we wanted to determine the necessity of Hsc66 function during cold shock survival. We monitored the A_{600}s of a wild-type culture (THK38) and an isogenic *hscA2-cat* insertion mutant (ML59) following cold shock to determine if the absence of Hsc66 affected survival or the rate of recovery following cold shock. ML59 showed a slightly lower A_{600} in stationary phase than THK38. In comparison with the severe effects of a *dnaK* mutation on heat shock survival rates, which include slow growth, extreme temperature sensitivity, defective chromosomal segregation, and decreased viability (4, 5), the effects of the *hscA2-cat* allele on cold shock survival were minimal. The function of Hsc66 during cold shock adaptation may be duplicated by the product(s) of another, unidentified cold-inducible gene. If there is functional redundancy in the cold shock response, the absence of Hsc66 in ML59 would not have a pronounced effect on cold shock survival, as seen in this study.

Contrary to the unaffected growth rate of a cold-shocked *hscA* mutant, this mutation did affect the cold shock protein profile. We examined the proteins produced 3 h following a cold shock in THK38 (*hscA*$^+$) and ML59 (*hscA2-cat*) cells. The expression of at least five other proteins was altered in ML59. This suggests that Hsc66 is required for expression or proper processing of some proteins following a cold shock. In the absence of Hsc66, the levels of some proteins increased, while the levels of other proteins were not maintained.

What function does Hsc66 perform to warrant its cold shock induction? Hsc66 is a member of the Hsp70 class of proteins and has 62% similarity to *E. coli* DnaK. Hsp70 proteins act as molecular chaperones, which bind to nascent, misfolded, or aggregated proteins. Upon binding, molecular chaperones can assist these proteins in achieving or retaining a particular conformation or assist in delivery to a protein secretion or degradation mechanism (reviewed in reference 2). Furthermore, Hsp70 proteins are typically induced under stress conditions. This study has shown that Hsc66 is induced under the cellular stress of cold shock. If homology predicts function, then Hsc66 may act as a molecular chaperone in the cold shock stimulon in a manner analogous to the way that DnaK acts as a molecular chaperone in the heat shock regulon. It is interesting that one other cold-inducible Hsp70 protein has been identified, in spinach (*Spinacia oleracea*) (1).

We have also identified another cold-inducible gene, the *hscB* gene, immediately upstream of *hscA*. *hscB* is a member of the *dnaJ* superfamily (23). As Hsp70 proteins typically require additional cellular cofactors, such as the requirement of DnaJ for proper DnaK function (11, 17, 27), it is logical to conclude that if Hsc66 does act as a molecular chaperone, it may require other cold-inducible cofactors, such as the product of *hscB*, for proper functioning.

Hsp70 proteins are typically induced under stress conditions. More accurately, though, Hsp70 proteins are induced under conditions that cause an increase in protein unfolding. Although protein aggregation is commonly thought to decrease at lower temperatures, to our knowledge, the rate of protein unfolding following cold shock in *E. coli* has not been characterized, nor have we presented any evidence that Hsc66 functions to alter protein unfolding under any environmental conditions. Addressing these concerns is the next step in characterizing Hsc66 function in the cold shock response.

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