Massive presence of the *Escherichia coli* ‘major cold-shock protein’ CspA under non-stress conditions

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The most characteristic event of cold-shock activation in *Escherichia coli* is believed to be the *de novo* synthesis of CspA. We demonstrate, however, that the cellular concentration of this protein is $\geqslant 50 \mu M$ during early exponential growth at 37°C; therefore, its designation as a major cold-shock protein is a misnomer. The *cspA* mRNA level decreases rapidly with increasing cell density, becoming virtually undetectable by mid-to-late exponential growth phase while the CspA level declines, although always remaining clearly detectable. A burst of *cspA* expression followed by a renewed decline ensues upon dilution of stationary phase cultures with fresh medium. The extent of cold-shock induction of *cspA* varies as a function of the growth phase, being inversely proportional to the pre-existing level of CspA which suggests feedback autorepression by this protein. Both transcriptional and post-transcriptional controls regulate *cspA* expression under non-stress conditions; transcription of *cspA* mRNA is under the antagonistic control of DNA-binding proteins Fis and H-NS both in vivo and in vitro, while its decreased half-life with increasing cell density contributes to its rapid disappearance. The *cspA* mRNA instability is due to its 5' untranslated leader and is counteracted in vivo by the cold-shock DeaD box RNA helicase (CsdA).

*Keywords*: DEAD box RNA helicase/Fis/H-NS antagonism/growth cycle/mRNA degradation/RNA chaperone

Introduction

A specific set of proteins, involved in a variety of essential functions, is induced when mesophilic bacteria are transferred below 20°C (Jones et al., 1987; Jones and Inouye, 1994; Graumann et al., 1997; Graumann and Marahiel, 1998; Yamanaka et al., 1998). The most characteristic of these cold-shock proteins in *Escherichia coli* is CspA, designated the ‘major cold-shock protein’ because its rapid and massive *de novo* expression is considered essential for cold adaptation (Jones et al., 1987; Goldstein et al., 1990; Tanabe et al., 1992; Jones and Inouye, 1994).

Indeed, CspA stimulates transcription of at least two cold-shock genes, *hns* (La Teana et al., 1991) and *gyrA* (Jones et al., 1992), and expression in *E.coli* of its *Bacillus subtilis* homologue (CspB) induced a cold shock-like response at 37°C (Graumann and Marahiel, 1997). CspA is homologous to several bacterial and eukaryotic single-strand nucleic acid-binding Y-box proteins (Sommerville and Ladomery, 1996; Graumann et al., 1997; Graumann and Marahiel, 1998; Yamanaka et al., 1998), binds single-stranded DNA and RNA (Graumann and Marahiel, 1994; Schindelin et al., 1994; Jiang et al., 1997) and stimulates *cspA* mRNA translation (Brandi et al., 1996). In spite of the extensive information available on the structure and properties of CspA, its role remains elusive and the expectation of a cold shock-specific function (e.g. anti-freeze function) may have been misleading. In fact, here we present evidence that the presence of CspA is not restricted to cold-shocked cells but that it represents almost 1% of total soluble proteins in cells in early exponential growth at 37°C, which strongly suggests an RNA chaperone role for CspA. We also found that *cspA* expression at 37°C and its cold induction are growth cycle dependent and that, under non-stress conditions, *cspA* is regulated by the antagonistic effects of Fis and H-NS on transcription, variations of *cspA* mRNA stability and, possibly, auto-repression.

Results

While studying the mechanism of *cspA* induction during cold shock in *E.coli*, we realized that, contrary to the reported literature, the steady-state levels of both *cspA* mRNA and CspA protein, as detected by Northern and Western blotting, respectively, were very high during early exponential growth at 37°C in cells that were never exposed to low temperature. After these data were obtained, the existence of several genes encoding CspA homologues was reported (for reviews see Graumann et al., 1997; Graumann and Marahiel, 1998; Yamanaka et al., 1998); in particular, eight additional Csp proteins (CspB, CspC, CspD, CspE, CspF, CspG, CspH and CspI) were identified in *E.coli*. In the light of the discrepancy of our data with previous reports (Goldstein et al., 1990; Jones and Inouye, 1994) and since only CspB and CspG of the above proteins were reported to be cold shock-inducible, it became imperative to demonstrate unambiguously that what we had detected in non-cold-shocked cells were indeed *cspA* mRNA and CspA. Thus, we used as probes both the entire *cspA* gene that could have cross-hybridized with homologous transcripts and *cspA*-specific oligonucleotide probes as well as oligonucleotide probes specific for each of the *cspA* homologues as controls. All these experiments gave consistent results, allowing us to conclude that non-cold-shocked cells contain a substantial amount of *cspA* transcript during early exponential growth at 37°C, which declines to become almost undetectable by mid-to-late exponential growth (Figure 1A). Furthermore,
when stationary phase cells containing virtually no cspA mRNA are diluted with fresh medium, the level of this transcript increases rapidly and extensively before declining again (Figure 1B). It should be noticed that, given the extremely short half-life (≤10 s) of the cspA mRNA at 37°C (Goldenberg et al., 1996), the steady-state levels of this RNA can be taken as a reliable measure of the activity of the corresponding promoter. Results consistent with those shown in Figure 1A and B were obtained when the cspA transcript was detected by extension of a cspA-specific oligonucleotide primer (Figure 1C). These experiments also demonstrated that one and the same cspA promoter is used throughout the growth cycle at 37°C (compare samples taken from cells in early, middle and late exponential growth; Figure 1C) and after cold shock. The presence of cspA transcript in non-cold-stressed cells does not ensure per se that CspA is also present, since the mRNA could be functionally inactive or the translational apparatus of these cells could be incapable of using it. Thus, the levels of CspA were determined by Western blotting followed by immunodetection and quantification; to ensure the resolution of this protein from its size and sequence homologues, the electrophoresis prior to Western blotting was performed under various conditions of pH, in the presence or absence of urea. Consistent with previous results, these experiments (Figure 1D) demonstrate that a very large amount of CspA is present during early exponential growth and that a
and CspA (explained by autorepression of cspA its maximum level detected at 37°C. Thus, the cold-shock of total soluble protein is such that the net increase maximum level attained by CspA after cold shock (to-late log cells (Figure 2B). In all cases, however, the maximum increment (30-fold) was seen in mid-
level of this protein in cells at different stages of growth (Figure 2A). The total amount of cold shocked during mid- and late-exponential growth namely a 25- to 50-fold increase, was observed in cells early exponential growth at 37°C. As with cspA mRNA, the highest level attained by the same mRNA during induction of cspA (Goldstein et al., 1991) and, at least during cold shock, of CspA (La Teana et al., 1991).

To investigate the possible existence of similar regulatory loops in cspa and hns, three different constructs were used; these contained a 170 bp (–90/+80), a 310 bp (–145/+165) and a 590 bp (–425/+165) fragment of the cspa promoter fused to a promoterless cat gene (Goldenberg et al., 1997) and were designated cspa170::cat, cspa310::cat and cspa590::cat, respectively. The expression of these fusions was analysed in vitro in the presence or absence of DNA-binding proteins Fis and H-NS and in vivo under different growth conditions. In agreement with the finding that cspa is efficiently transcribed in vivo at 37°C (Figure 1A), preliminary tests showed that at this temperature the cspa promoter is also active in vitro (more active than at 10 or 15°C). Additional experiments showed that transcription from the cspa promoter is stimulated by Fis and inhibited by H-NS. Under the experimental conditions described in the legend for Figure 3A, stimulation by Fis is 2.5- to 3-fold but it can be ≥6-fold if the amount of RNA polymerase is halved (from 0.2 to 0.1 unit). Furthermore, since transcription from cspa170::cat and cspa590::cat is stimulated to the same extent by Fis, the target of this protein must lie within the shorter cspa fragment, in agreement with the localization of all Fis binding sites within this DNA region (A.Brandi and M.Falconi, unpublished data). Inhibition by H-NS, on the other hand, was somewhat stronger on cspa590::cat transcription compared with cspa170::cat (Figure 3B), presumably because cspa170::cat lacks an upstream H-NS binding site (A.Brandi and M.Falconi, unpublished data). As in the case of hns (Falconi et al., 1996), the amount of Fis required to elicit stimulation is ~3-fold lower compared with the amount of H-NS required for inhibition, indicating a somewhat stronger affinity of Fis for the upstream region of cspa. These in vitro effects of Fis and H-NS on the activity of the cspa promoter (Figure 3A and B) are consistent with the findings that, in

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Fig. 2. Relative increase of cspa mRNA and CspA and total levels of CspA in cells cold shocked at different phases of growth. E.coli MRE600 cells growing in LB medium at 37°C were shifted to 10°C at the indicated cell densities. At the indicated times after cold shock, samples were withdrawn from each culture for the extraction of total RNA and total protein which were processed and analysed as described in the legend for Figure 1. The results obtained as a function of the time of cold shock shown in the abscissa are plotted as the increase (-fold) of cspa mRNA (A) and CspA (B) with respect to the basal level (taken as 1) detected before cold shock (time 0). The cell densities of the cultures were A620 = 0.15 (Δ); 0.3 (Ο); 0.6 (△); 1.0 (■) and 1.6 (▲) for the experiment of (A) and A620 = 0.2 (□); 0.6 (▲) and 2.0 (●) for the experiment of (B). The total level of CspA in cell samples taken at A620 = 0.2 (■); 0.6 (▲); 1.2 (●) and 2.0 (●) is reported in (C) as AU.

Comparison of the promoter and upstream regions of cspa and hns revealed the presence of several sequence elements (boxes) with different degrees of homology within 300 bp upstream of the translational starts. This suggests that at least some of the mechanisms controlling cspa expression might be the same as those controlling hns whose transcription is under negative control of

H-NS (Dersch et al., 1993; Falconi et al., 1993; Ueguchi et al., 1993), positive control of Fis (Falconi et al., 1993, 1996) and, at least during cold shock, of CspA (La Teana et al., 1991).
cells growing out of stationary phase, the steady-state levels of $cspA$ transcript (not shown) and CspA (Figure 3C) were substantially lower (in fis') and higher (in hns') compared with the wild-type strain, while the hns'/fis' double mutant and wild-type cells contained essentially the same amount of both transcript and protein (Figure 3C). These results indicate that Fis and H-NS also have an antagonistic influence on $cspA$ expression in vivo.

In search of additional elements that could account for the growth phase-dependent variations in $cspA$ expression, the activity of the cat reporter gene was measured in cells carrying the above-mentioned $cspA::cat$ fusions. At all cell densities, the levels of cat transcript (Figure 4A) and chloramphenicol acetyltransferase (CAT) activity (Figure 4B) were substantially higher when expressed from $cspA_{170}::cat$ compared with the other two constructs. Furthermore, the CAT activity, which remained essentially constant throughout cell growth when expressed from $cspA_{170}::cat$, declined sharply with increasing cell density when expressed from the other two constructs (Figure 4B).

Since transcriptions from the longest and shortest $cspA::cat$ construct (Figure 3A and B) as well as from $cspA_{310}::cat$ (not shown) are equally sensitive to Fis activation and similarly sensitive to H-NS inhibition, the different behaviour in vivo of these constructs is probably due to post-transcriptional events. That this is the case is shown by the evidence (Figure 5A and B) that the $cspA_{170}::cat$ transcript ($t_{1/2} \geq 90 \text{s}$) is substantially more stable than the $cspA_{590}::cat$ transcript whose half-life ($t_{1/2} < 30 \text{s}$) is similar to that of $cspA$ mRNA (10 s) (Goldenberg et al., 1996). Furthermore, the half-life of the $cspA_{590}::cat$ transcript decreases from early- (Figure 5A) to mid- (Figure 5B) exponential growth, so that the difference in chemical stability between the two RNAs is clearly enhanced with increasing cell density. As all the transcripts contain the same cat fusion and since cat mRNA stability increases with the age of the culture (Kuzj et al., 1998), the $cspA$ portion of the transcript is likely to be responsible for the observed difference in stability. Indeed, $cspA_{170}::cat$ differs from the other two constructs in that it lacks approximately half of the long 5' untranslated leader (5' UTR) of $cspA$ mRNA. This 5' UTR, endowed with extensive self-complementarity, could be the target of a nuclease whose level increases with the age of the culture (see Discussion). The interpretation that the secondary structure of its 5' UTR is responsible for the lability of $cspA$ mRNA is compatible with the finding that, compared with the controls, the levels of $cspA$ mRNA are higher in cells (transformed with pUCDeaD) that carry extra copies of the gene encoding the DEAD-box RNA helicase (Iost and Dreyfus, 1994; Figure 5C). This difference is small (i.e. \(\sim 1.5\)-fold) during early-to-mid exponential growth but becomes substantially larger (\(\approx 3-4\) times) when, as seen in Figure 5C, the cells harbouring pUCDeaD accumulate increasingly higher levels of RNA helicase. Additional experiments confirmed that, as expected, the higher levels of $cspA$ mRNA in cells containing higher levels of DeaD-box RNA helicase are due to an increased half-life of $cspA$ mRNA.

**Discussion**

In this article we demonstrate that, upon growth out of stationary phase at 37°C, *E. coli* cells contain an extremely large amount (\(> 100,000\) copies) of CspA whose level, though diminishing with increasing cell density, remains clearly detectable throughout the growth cycle. Furthermore, we show that the level of this protein increases during cold shock in a strongly growth phase-dependent way; however, its level did not increase more than 2- to 3-fold above the maximum level detected during early exponential growth at 37°C, comparable with the cold-shock induction (2- to 10-fold) reported for all other cold-shock genes (Jones et al., 1987). Previous studies in which cold-shock response was induced exclusively in cultures in mid-to-late exponential growth and analysed quantitatively through the incorporation of radioactive precursors without any correction for possible changes in the pool sizes, led...
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Fig. 4. Growth phase-dependent expression of CAT and steady-state levels of cat transcript in cells carrying different types of cspA::cat fusions. E.coli cells carrying the cspA170::cat (■), cspA310::cat (Δ), and cspA590::cat (○) fusions were grown in LB medium at 37°C. At the indicated cell densities, samples were harvested and processed for total protein and RNA extraction. The levels of CAT mRNA (A) were determined by hybridizing Northern blots of 4 μg of total RNA with a radioactive cat probe, quantified by Molecular Imager and expressed as arbitrary units after normalization for the levels of 16S rRNA in the same samples. The CAT activity expressed by each type of cells (B) was determined (Goldenberg et al., 1997) and reported as percentage of the activity determined in the same type of cells in early exponential growth (A$_{620}$ = 0.25). This normalized activity (taken as 100%) corresponds to 51.0, 19.6 and 22.6% butyrylated chloramphenicol formed by 4 μg of total protein extract of cells harbouring cspA170::cat, cspA310::cat and cspA590::cat, respectively.

Fig. 5. Chemical stability of cspA::cat transcripts with or without the intact 5’ UTR of cspA and influence of DeaD (CsdA) RNA helicase on the steady-state levels of cspA mRNA. E.coli cells carrying cspA590::cat (○) or cspA170::cat (■) fusions were grown at 37°C in LB medium containing ampicillin. At A$_{620}$ = 0.25 (A) or at A$_{620}$ = 1.0 (B), rifampicin (200 μg/ml) was added; aliquots of the cultures were harvested and the levels of cat transcript determined as described in Figure 1. The CAT mRNA expressed from each type of fusion and remaining intact at the indicated times is plotted as the percentage of the amount of the same mRNA present at the time of rifampicin addition (t$_0$). The initial levels were almost identical in the cultures taken at A$_{620}$ = 0.25, while at A$_{620}$ = 1.0 the level of the cspA170::cat transcript was ~3 times higher than that expressed from cspA590::cat. (C) Level of the DeaD (CsdA) RNA helicase accumulating, during growth, in E.coli JM109 cells harbouring a pUC18 vector carrying E.coli deaD encoding the DeaD box RNA helicase (●) and the steady-state levels of cspA mRNA (quantified as described in Figure 3) detected in the same cells (■) and in control cells harbouring pUC18 vector without insert (Δ). The ratio of the normalized levels of cspA mRNA in cells with or without extra copies of the RNA helicase gene is also shown (□).

to the conclusion that cold shock induces cspA de novo. The present findings are in contrast with these previous reports and indicate that the denomination ‘major cold-shock protein’ attributed to CspA (Goldstein et al., 1990; Jones and Inouye, 1994) is, in a strict sense, a misnomer. More importantly, however, our findings imply that the function of CspA cannot be restricted to a specific role during cold adaptation, but must include activities required under non-stress conditions, mainly during active growth out of stationary phase. In light of its extraordinarily high abundance detected during early exponential growth, which correlates well with the overall transcriptional activity of the cell, and considering the low level of sequence selectivity with which CspA interacts with nucleic acids (Graumann and Marahiel, 1994, 1998; Graumann et al., 1997; Yamanaka et al., 1998), it seems reasonable to surmise that this protein may fulfill a very general role of chaperoning nascent mRNAs and/or rRNAs, thereby sustaining active growth by favouring transcription, translation and/or ribosome assembly. The stimulation of cspA transcription by Fis and the inhibition by H-NS, two factors acting as antagonistic sensors of the metabolic state of the cell, is in full agreement with this hypothesis. Finally, CspA could also act as a general transcriptional stimulator stabilizing open complex formation or promoter clearance by binding to single-stranded DNA at 37°C as it probably does on some genes during cold shock (Brandi et al., 1994).
Regardless of whether these hypotheses are correct, the reason for the existence of several CspA homologues in E. coli cells, two of which (CspB and CspG) are induced by cold shock and one (CspD) by entry into stationary phase (Yamanaka and Inouye, 1997), remains mysterious.

More than one mechanism is involved in triggering the cold-shock expression of cspA. In fact, while a factor binding upstream of the cspA core promoter may activate transcription (Tanabe et al., 1992; Jones and Inouye, 1994), post-transcriptional events such as stabilization of the cspA mRNA (Tanabe et al., 1992; Brandi et al., 1996; Goldenberg et al., 1996) and its selective translation by the cold shock-modified translational apparatus ensures the massive synthesis of CspA (Brandi et al., 1996; Goldenberg et al., 1997).

We have shown here that, at 37°C, cspA expression is regulated by antagonistic Fis/H-NS transcriptional control and by post-transcriptional changes in cspA mRNA stability. These mechanisms resemble only in part those responsible for cspA regulation during cold shock. In fact, unlike the variations in mRNA stability, which are equally important at 37°C and following cold shock, the Fis/H-NS antagonism that accounts, to a large extent, for the growth phase-dependent fluctuation of cspA expression plays no role during cold shock.

The demonstration that cold-shock induction of cspA is inversely proportional to the pre-existing level of CspA, the detection of a weak interaction between CspA and matrix-bound cspA promoter (A. Brandi, unpublished data) and other data in the literature (Bae et al., 1993, 1996). cspA box RNA helicase CsdA can stabilize the transcript, our data indicate that the 3′ UTR of the cspA mRNA, together with a nuclease whose activity increases with increasing cell density, play a key role in the control of the half-life of this RNA. It has been shown, in fact, that deletion of approximately half of the 159 nucleotides of the 5′ UTR or the presence of a higher cellular level of the DEAD-box RNA helicase CsdA can stabilize the cspA transcript. Both conditions are expected to interfere with the formation of the extended stem–loop structure predicted for the 5′ UTR of cspA (Etchegaray et al., 1996), which could represent the target of RNase E (Cohen and McDowall, 1997; Fang et al., 1997) whose activity increases with cell density. It is relevant to recall, in this connection, that CsdA is one of the early proteins (if not the earliest) induced by cold shock (Jones et al., 1996; Goldenberg et al., 1997) whose accumulation could also stabilize cspA mRNA at the onset of cold shock.

The level of cspA mRNA declines rapidly with increasing cell density and becomes virtually undetectable by mid-log phase (Figure 1A) indicating that, under these conditions, synthesis of cspA mRNA, if any, occurs at a lower rate compared with its degradation. This is consistent with the extremely short (≈10 s) half-life reported for cspA mRNA at 37°C (Goldenberg et al., 1996). In parallel, the CspA level is also reduced (Figure 1D) at a rate compatible with dilution of this protein through cell doubling in the absence of de novo synthesis and of substantial degradation.

In summary, fluctuations of CspA level during cell growth at 37°C can be rationalized as follows. In fresh cultures, when growth rate is maximal, a high level of CspA expression is ensured by a combination of (i) favourable gene dosage effect (cspA maps near oriC), (ii) the high concentration of its transcriptional activator Fis and (iii) higher stability of cspA mRNA due to lower RNase activity. With increasing cell density, the disappearance of Fis, accompanied by the accumulation of the transcriptional repressor H-NS, causes the decline (or complete cessation) of cspA transcription, while the cspA transcript disappears rapidly due to an increase in the rate of its degradation. Finally, it is interesting to note that the transcriptional regulatory loops described here for cspA may be of more general relevance since other cold-inducible genes were found to be subjected to similar types of regulation (A. Brandi, unpublished observations).

Materials and methods

Escherichia coli strains used were MRE600, DH5, JM109 (Sambrook et al., 1989) and four strains kindly provided by Dr W. Messer (Berlin), namely, WM 2482 (wild type), WM 2648, WM 2649 and WM 2650 carrying hns null, fis null and hns/fis double null mutations, respectively. Total RNA was extracted as described previously (La Teana et al., 1991), except that hot phenol and chloroform:isoamyl alcohol (24:1) extractions were performed in place of purification in CsCl. Aliquots of RNA (8 μg) were subjected to electrophoresis, blotting and hybridization as described (Brandi et al., 1996). The blots were probed with the cspA (Brandi et al., 1996) or the cat gene (Goldenberg et al., 1997) or with 5′-end-labelled oligonucleotides (Sambrook et al., 1989). The oligonucleotide specific for cspA (5′-CTTCGATGGTGAAGGACACT-3′), unlike the other probes, was hybridized at 42°C in oligonucleotide prehybridization solution (Sambrook et al., 1989) with 0.2× Denhardt’s solution in place of non-fat dried milk and washed with 2× standard saline citrate (SSC) for 5–10 min at 42°C. An rrrB probe, excised from pKK3535 by digestion with HindIII and 32P-labelled by random primer reaction served as internal control.

CspA was quantified in cells disrupted by sonication in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.8 mM KH2PO4) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 6 mM 2-mercaptopetoanol. Total protein (5–10 μg, as determined in Bradford, 1976) was subjected to 15% PAGE under non-denaturing conditions in a non-dissociating discontinuous system at high pH (Hames and Rickwood, 1981) followed by electroblotting in 50 mM Tris–HCl pH 8.3, 80 mM glycine, 0.04% SDS and 20% methanol onto a cellulose nitrate membrane using the Sammy Dry System (Schleicher & Schuell). CspA was detected and quantified essentially as described (Brandi et al., 1996).

Primer extension analysis was carried out on 8 μg samples of total RNA using 2 pmol of a 20-mer oligonucleotide corresponding to nucleotides 174–193 of the template strand as primer. The reaction served as internal control.

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from 10 ml aliquots of culture harvested just before (t0) and 0.5, 1, 2, 3 and 4 min after rifampicin addition (200 μg/ml). The RNAs were quantified as described above.

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