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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 ⩾50 μM 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, autorepression.

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,

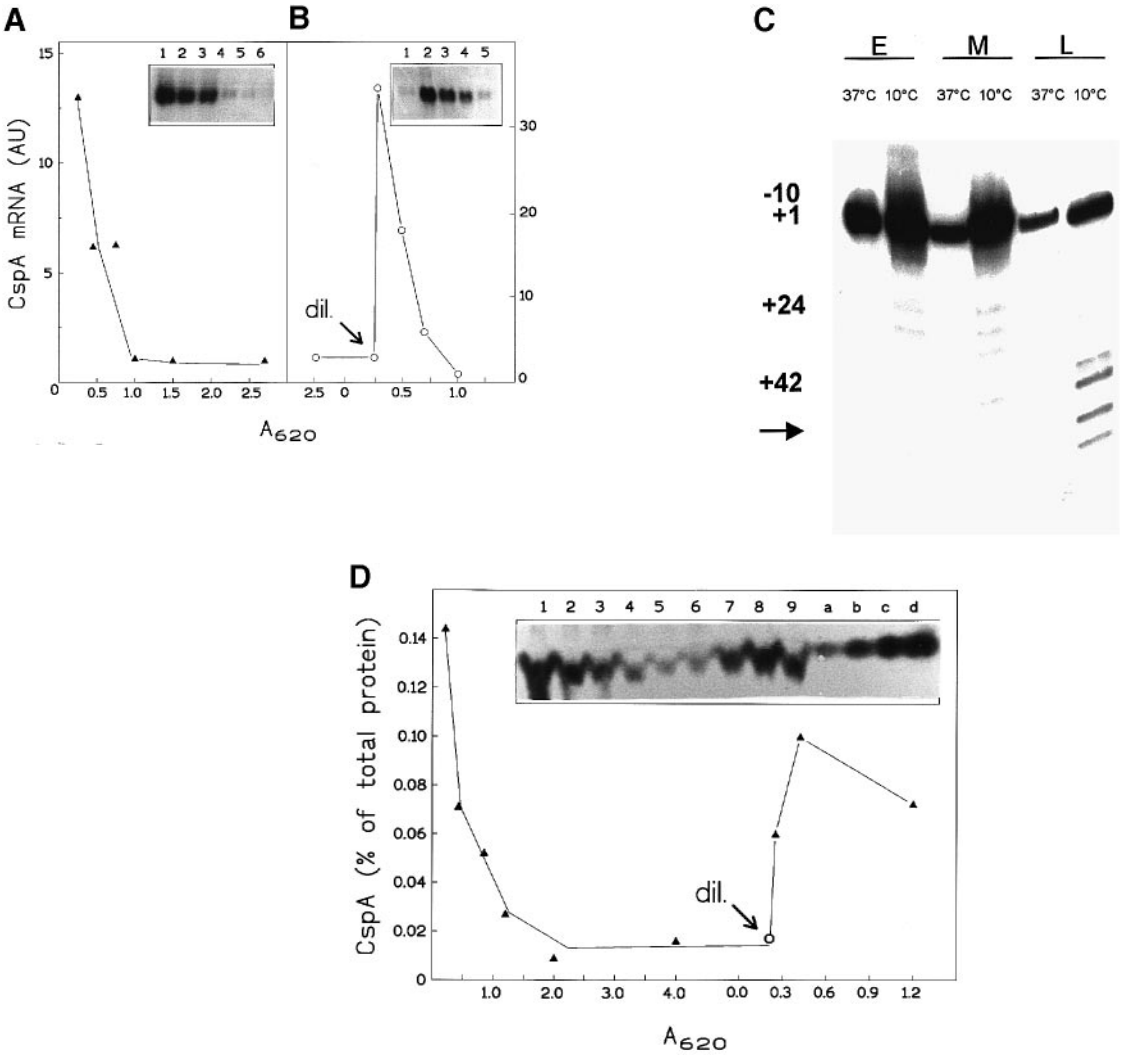

Fig. 1. Steady-state levels of cspA mRNA and CspA as a function of cell growth. Aliquots of *E.coli* MRE600 cells growing in LB medium at 37°C were taken at the indicated cell densities before (**A**) and after (**B**) 10-fold dilution of a stationary phase culture. After extraction and electrophoresis of total RNA, the *cspA* transcript present in each sample was detected by hybridization of the Northern blots with a radioactive *cspA* DNA probe followed by autoradiography (see inserts); the levels of the transcript were quantified by Molecular Imager (Bio-Rad) and expressed in the ordinate as arbitrary units (AU). Analysis of the transcriptional start point of *cspA* by primer extension (as described in Materials and methods) is presented in (**C**) and refers to *E.coli* MRE600 cells harvested at different stages of growth in LB medium at 37°C (E, A_{620} = 0.15; M, 0.6; and L, 1.5) before (lanes marked 37°C) or after 90 min cold shock at 10°C (lanes marked 10°C). The numbers on the left refer to positions on the DNA sequence, the start site being at position +1 whereas the arrow indicates the position of a second hypothetical start site (Tanabe *et al*., 1992). The total proteins extracted from cells obtained under similar conditions were subjected to electrophoresis and Western blotting. The CspA levels in cells during growth in LB medium at 37°C and following dilution of a stationary phase culture with fresh medium were determined (**D**) by densitometric analysis of the Western blots (see insert). The arrows indicate the points at which the cultures were diluted. The numbered electrophoretic lanes [inserts of (A), (B) and (D)] correspond to samples taken in sequential order at the indicated cell densities. Lanes in which increasing amounts of purified CspA had been loaded are indicated by the lower case letters [insert of (D)]. The quantification of CspA by this method was found to be linear up to at least 40 ng. Further details are given in Materials and methods.

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

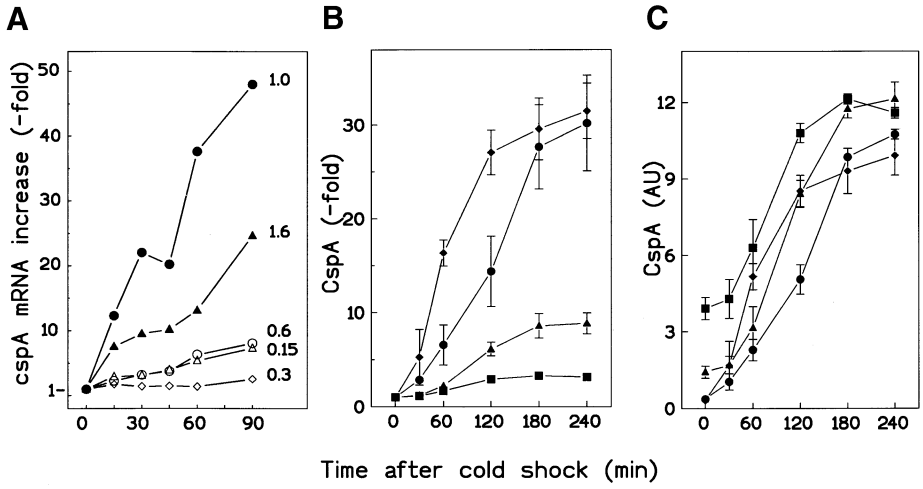

Fig. 2. Relative increase of *cspA* mRNA and CspA and total levels of CspA in cells cold shocked at different phases of growth. *E.coli* MRE600 cells growing in LB medium at 37°C were shifted to 10°C at the indicated cell densities. At the indicated times after cold shock, samples were withdrawn from 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 A_{620} = 0.15 (Δ); 0.3 (◇); 0.6 (○); 1.0 (●) and 1.6 (▲) for the experiment of (A) and A_{620} = 0.2 (■); 0.6 (▲); 1.2 (●) and 2.0 (◆) for the experiment of (B). The total level of CspA in cell samples taken at A_{620} = 0.2 (■); 0.6 (▲); 1.2 (●) and 2.0 (◆) is reported in (**C**) as AUs.

clearly detectable amount remains throughout growth at 37°C. Thus, our data are inconsistent with the notion that *cspA* is expressed almost exclusively during cold shock (Goldstein *et al*., 1990; Jones and Inouye, 1994) since in cultures at A_{620} = 0.2 (~1.5×10^7 cells/ml) CspA represents ⩾0.7% of total soluble protein, corresponding to ~5–6×10^5 molecules/cell (Goodsell, 1991).

The levels of *cspA* mRNA and CspA were also measured at different times following cold shock in cells harvested at different stages of growth. These experiments (Figure 2A and B) demonstrate that the growth phase of the culture can influence also the extent of cold-shock induction of *cspA*. Notwithstanding the large differences in the pre-shock *cspA* mRNA levels, the highest induction, namely a 25- to 50-fold increase, was observed in cells cold shocked during mid- and late-exponential growth (Figure 2A). The total amount of *cspA* mRNA, however, normalized for the amount of 16S rRNA, did not increase after cold shock more than 2- to 3-fold in comparison with the highest level attained by the same mRNA during early exponential growth at 37°C. As with *cspA* mRNA, the cold induction of CspA also depended on the initial level of this protein in cells at different stages of growth and the maximum increment (30-fold) was seen in mid-to-late log cells (Figure 2B). In all cases, however, the maximum level attained by CspA after cold shock (≈2% of total soluble protein) is such that the net increase attributable to cold stress is no more than 2.5-fold above its maximum level detected at 37°C. Thus, the cold-shock inducibility of *cspA* expression is inversely related to the pre-shock level of CspA, a relationship which could be explained by autorepression of *cspA* (see Discussion).

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).

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

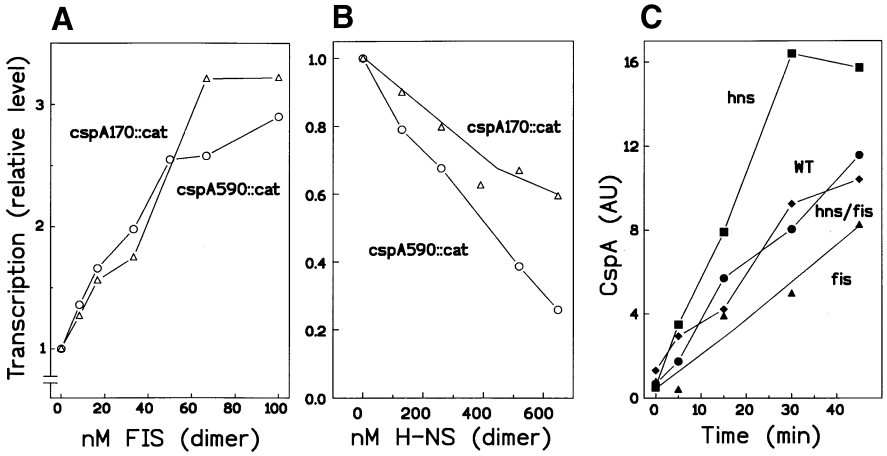

Fig. 3. Effect of Fis and H-NS on cspA expression *in vitro* and *in vivo*. Activity of the *cspA* promoter *in vitro* in the presence of Fis (**A**) and H-NS (**B**); the templates for *in vitro* transcription were pKK232-8 vectors carrying *cspA170::cat* (Δ) or *cspA590::cat* (○). After 10 min preincubation at 37°C with the indicated amounts of either Fis or H-NS, the reactions were carried out at the same temperature for 15 (A) or 12 (B) min using 0.2 units of RNA polymerase as described in Materials and methods. (**C**) *In vivo* levels of CspA in wild type (◆), *fis*⁻ (▲), *hns*⁻ (■), *hns*⁻/*fis*⁻ (●) background. *Escherichia coli* cells were grown to saturation at 37°C in LB medium (with antibiotic additions as appropriate), diluted 20-fold with fresh medium and allowed to resume growth for the indicated times before samples were withdrawn and processed for total protein extraction and CspA quantification. The CspA levels present in each sample are normalized for the total amount of protein and are expressed as AUs. Further details are given in Figure 1 and in Materials and methods.

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 *cspA170::cat* compared with the other two constructs. Furthermore, the CAT activity, which remained essentially constant throughout cell growth when expressed from *cspA170::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 *cspA310::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 *cspA170::cat* transcript ($t_{1/2}$ ⩾90 s) is substantially more stable than the *cspA590::cat* transcript whose half-life ($t_{1/2}$ <30 s) is similar to that of *cspA* mRNA (10 s) (Goldenberg *et al.*, 1996). Furthermore, the half-life of the *cspA590::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, *cspA170::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. ≈1.5-fold) during early-to-mid exponential growth but becomes substantially larger (≈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

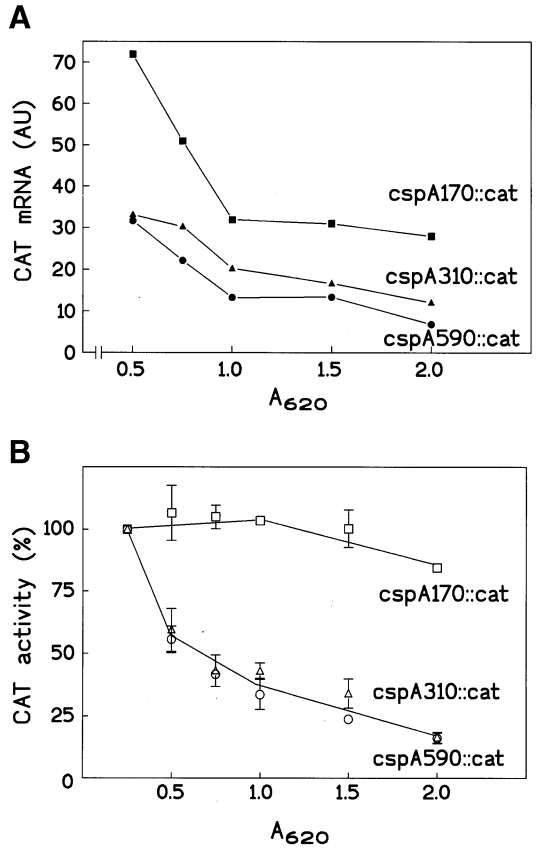

Fig. 4. Growth phase-dependent expression of CAT and steady-state levels of *cat* transcript in cells carrying different types of *cspA::cat* fusions. *E.coli* cells carrying the *cspA170::cat* (□; ■), *cspA310::cat* (△; ▲) and *cspA590::cat* (○; ●) fusions were grown in LB medium at 37°C. At the indicated 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.

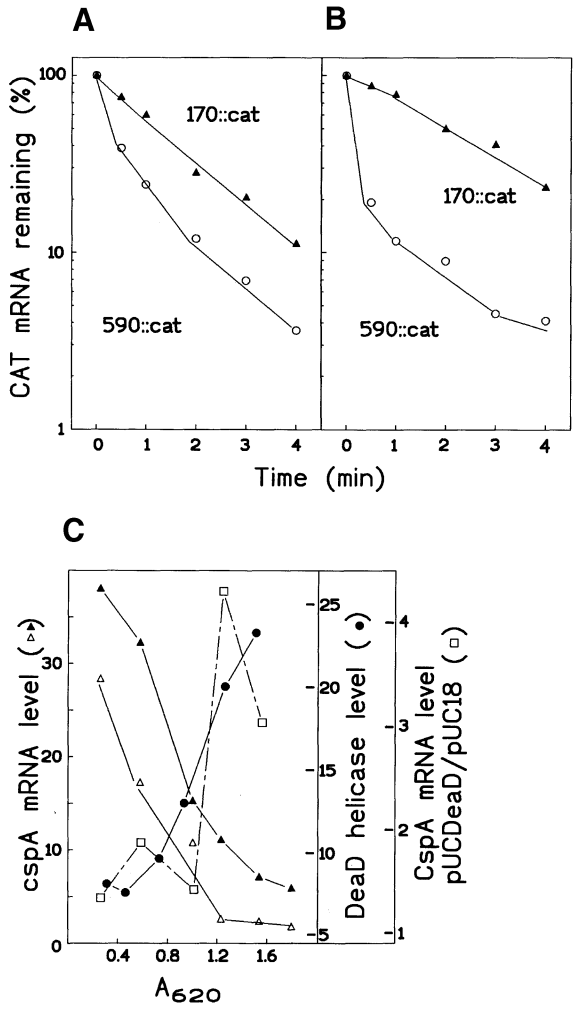

Fig. 5. Chemical stability of *cspA::cat* transcripts with or without the intact 5′ UTR of *cspA* and influence of DeaD (CsdA) RNA helicase on the steady-state levels of *cspA* mRNA. *E.coli* cells carrying *cspA590::cat* (○) or *cspA170::cat* (▲) fusions were grown at 37°C in LB medium containing ampicillin. 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 fulfil 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*., 1997; Graumann *et al*., 1997) suggest that *cspA* may be subjected to autorepression. An obvious target for this type of regulation could be the CCAAT sequence present between the –10 and –35 *cspA* promoter elements, but several experiments have failed to prove this hypothesis (Goldenberg *et al*., 1997). Nonetheless, the existence of a triple control by H-NS, Fis and CspA of *cspA* expression is remarkably similar (but for the opposite effect of CspA) to that found in *hns* regulation (La Teana *et al*., 1991; Falconi *et al*., 1993, 1996).

Regarding the extreme and somewhat growth phase-dependent instability of the *cspA* transcript, our data indicate that the 5′ 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′-CTTTCGATGGTGAAGGACACT-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 *rrnB* probe, excised from pKK3535 by digestion with *Hin*dIII and ^{32}P-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 Na_2HPO_4, 1.8 mM KH_2PO_4) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 6 mM 2-mercaptoethanol. 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 (10 µl) was carried out at 42°C for 30 min with 2 units of AMV reverse transcriptase (Amersham) in the supplied buffer. The reaction products were analysed on 7% PAGE–urea gel in parallel with a *cspA* sequencing reaction performed by the Sanger dideoxy chain termination reaction using the same oligonucleotide primer (Sambrook *et al*., 1989).

In vitro transcription was performed in 25 µl reaction mixtures essentially as described in the figure legends and in Falconi *et al*. (1993). The amount of *cat* gene transcribed *in vitro* was determined (Goldenberg *et al*., 1997) by electrophoresis of the reaction products, followed by Northern blotting, hybridization with a radioactive *cat* DNA probe and quantification by a Bio-Rad Molecular Imager (model GS-250).

To determine the chemical stability of mRNA, total RNA was extracted

from 10 ml aliquots of culture harvested just before (t_0) 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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