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Role of *Escherichia coli cspA* promoter sequences and adaptation of translational apparatus in the cold shock response

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Abstract A shift in growth temperature from 37° C to 15° C leads to a dramatic increase in the level of CspA, the major cold shock protein of *Escherichia coli*. To investigate the molecular basis of this induction, we considered the relevance of transcriptional and post-transcriptional controls by analyzing the steady-state levels of transcripts and the expression of reporter genes in cells carrying a set of *cspA* promoter fragments of variable length fused to *lacZ* or *cat* genes. We demonstrate that: (i) the core *cspA* promoter (from –40 to +16) responds to cold shock and a mutation at –36 increases the relative activity of the promoter at low temperature by threefold; (ii) the sequences upstream of –40 have a positive effect on expression at 37° C, but no effect on the cold shock response; (iii) by virtue of their influence on mRNA stability, the downstream sequences (from +81 to +165) reduce expression at 37° C and increase the intensity of the cold shock response; (iv) mutations in the GCACATCA and CCAAT motifs, present at +1/–4 and between the –10 and –35 elements, respectively, do not affect the cold shock response of the *cspA* promoter; (v) following cold shock, a modification of the protein synthetic machinery takes place that allows preferential translation of *cspA* mRNA relative to the non-cold shock *cat* and *lacZ* mRNAs. The quantitatively modest transcriptional activation shown by the core promoter of *cspA* following cold shock suggests that transcriptional activation can significantly contribute to cold shock induction only when coupled to post-transcriptional controls, such as alterations in mRNA stability and of the translational apparatus.

Key words Cold shock · Core promoter activity · CCAAT sequence · Translational control · mRNA stability

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Introduction

Bacteria are well adapted to respond to instantaneous changes in temperature. A specific set of genes is induced when bacterial cells growing at 37° C are transferred to below 20° C (Jones et al. 1987; Jones and Inouye 1994). The most dramatic response is the rapid increase in *cspA* gene expression, whereby over 10% of total cellular protein synthesis is devoted to the synthesis of CspA (Jones et al. 1987; Goldstein et al. 1990; Tanabe et al. 1992). This protein, whose three-dimensional structure has recently been elucidated (Newkirk et al. 1994; Schindelin et al. 1994), contains 70 amino acids and displays a high degree of similarity to a domain in eukaryotic Y-box transcription factors (Wistow 1990; Wolffe et al. 1992). There are at least six CspA homologues in *E. coli*: CspB, CspC, CspD, CspE, CspF and CspG; only CspA, CspB and CspG are induced by cold (Lee et al. 1994; Yamanaka et al. 1994; Nakashima et al. 1996). CspA has been shown to stimulate moderately the translation of its own mRNA (Brandi et al. 1996) and the transcription of at least two *E. coli* cold shock genes, *hns* (La Teana et al. 1991) and *gyrA* (Jones et al. 1992). This activity is probably mediated by an interaction of CspA with a single-stranded form of the CCAAT sequences present in these genes, which presumably favors the formation of the open complex or promoter clearance by the RNA polymerase (Brandi et al. 1994).

More than one mechanism is likely to be involved in the cold-shock activation of *cspA*. In fact, several lines of

evidence suggest that *cspA* expression is regulated at the transcriptional (Tanabe et al. 1992; Jiang et al. 1993; Jones and Inouye 1994) and post-transcriptional (Brandi et al. 1996; Goldenberg et al. 1996) levels. In particular, a unidentified factor has been found to bind upstream of the core promoter of *cspA* (Tanabe et al. 1992), and the *cspA* mRNA was found to be extremely unstable in vivo at 37° C, with a half-life of about 10 s, and to be stabilized by cold shock (Brandi et al. 1996; Goldenberg et al. 1996). The instability of the *cspA* mRNA was to a large extent eliminated when the *cspA* promoter was fused to *lacZ* by manipulations which entailed the loss of part of the *cspA* 5′ untranslated leader (Goldenberg et al. 1996). Furthermore, it has been shown that even when transcriptional control by the natural promoter of *cspA* is bypassed, the preferential expression of CspA at low temperature still takes place, and that the translational apparatus of cold-shocked cells is modified so as to translate *cspA* mRNA in vitro with a selectively greater efficiency than the corresponding system from control cells (Brandi et al. 1996).

In this paper we show that a modest transcriptional activation following cold shock is an intrinsic property of the *cspA* core promoter; if boosted by post-transcriptional regulatory mechanisms, such as changes in mRNA stability and selective modification of the translational apparatus, for which we present additional evidence, this property of the *cspA* promoter could significantly contribute to the cold shock-dependent induction of CspA production.

Materials and methods

Construction of transcriptional *cspA* fusions

The *cspA-lacZ* fusions were obtained by PCR of phage 9F6 (602) DNA from the Kohara library (Kohara et al. 1987; Goldstein et al. 1990), and cloned into vector pIK86 as described previously (Goldenberg et al. 1996). All fusions were transferred by homologous recombination to phage λB299, which was inserted into the bacterial chromosome as a single copy within the *att* site of A6826, a strain carrying a *lac* deletion (for details see Giladi et al. 1995; Goldenberg et al. 1996).

The *cspA-cat* fusions were obtained by ligating DNA fragments of different lengths, derived from the *cspA* promoter region, to the promoterless *cat* gene in pKK232-8 (Pharmacia). Fusions –425/+165, –145/+165 and –90/+80 were obtained by subcloning the appropriate segments of the 1.2-kb *Pst*I-*Eco*RI fragment containing *cspA* isolated from λ phage 9F6 (Kohara et al. 1987) previously cloned into pTZ19R (Brandi et al. 1996). The –4335/+165 *cspA-cat* fusion was constructed by ligation of the 4.1-kb *Eco*RV-*Sma*I fragment excised from λ phage 9F6 (Kohara et al. 1987) to the 430-bp *Sma*I-(*Hpa*II)*Hin*dIII fragment from the –145/+165 *cspA-cat* fusion and subsequently inserting the reconstructed 4500-bp *Eco*RV-(*Hpa*II)*Hin*dIII fragment between the *Sma*I and *Hin*dIII sites in pKK232-8

Measurement of the RNA levels

The mRNA levels in *E. coli* A6826 cells carrying the *cspA-lacZ* fusions in the chromosome as single-copy prophages were determined by primer extension analysis using primer 1838 (5′-GGGATTAACTGCGCGTCG-3′) on total RNA extracts as previously described (Goldenberg et al. 1996). The *cat* mRNA level was determined by Northern blotting of total RNA (8 μg per lane) prepared from *E. coli* DH5 cells harvested at the indicated times; the RNA was subjected to formaldehyde-agarose gel electrophoresis and hybridization using a *cat* probe, derived from pCM7 (Pharmacia), and, as internal control, an rRNA probe, both ^{32}P-labelled by random priming. Further details are given in Brandi et al. (1996).

Measurement of enzyme activities

The β-galactosidase assays were carried out on cells processed as described (Goldenberg et al. 1996) according to the standard protocol (Miller 1972). For measurement of the chloramphenicol acetyltransferase (CAT) activity, cell extracts were prepared from 2-ml aliquots of cultures harvested at the indicated times. The cells were washed with 0.9% NaCl, resuspended in 0.25 M TRIS-HCl pH 8, and disrupted by sonication. The supernatant of the cell extract obtained after a 10-min incubation at 65° C and centrifugation, was used for CAT activity measurement using aliquots normalized for their protein content (Bradford 1976). The CAT activity was measured as the percentage of butyryl chloramphenicol, obtained by extraction with xylene, with respect to the total amount of [^{14}C]chloramphenicol in the reaction mixture (Seed and Sheen 1988).

Site-specific mutagenesis.

Both the CCAAT and GCACATCA motifs in the *cspA* promoter were mutagenized by two rounds of PCR using for each promoter fragment an appropriate pair of primers and DNA of phage 9F6 (602) from the Kohara library (Kohara et al. 1987) as template. In the first round, PCR was performed using one mutated primer and one of the flanking primers. In the second round, PCR was done using the second flanking primer and the purified product of the first round of PCR.

Results and discussion

We constructed a number of *cspA-lacZ* and *cspA-cat* transcriptional fusions (Fig. 1). The *cspA-lacZ* fusions (Fig. 1A) were transferred to phage λ by homologous recombination and each was then inserted into the bacterial chromosome, as a single copy within the *att* site, of the Δ*lac* strain A6826 following the published procedure (Giladi et al. 1995). The *cspA-cat* fusions (Fig. 1B) were constructed in pKK232-8 (Pharmacia) and transformed into *E. coli* DH5 cells as described (Falconi et al. 1993).

Activity of the P*cspA* fusions at 37° C

The steady-state levels of the transcripts derived from the various constructs, as well as the resulting enzyme activities (either β-galactosidase or chloramphenicol acetyltransferase) found at 37° C, are presented in Table 1. The first noteworthy feature of the results presented in this Table is that the activity of the reporter genes expressed at 37° C is always closely paralleled by the levels of the corresponding transcripts. With respect to the influence of the various manipulations, it can be

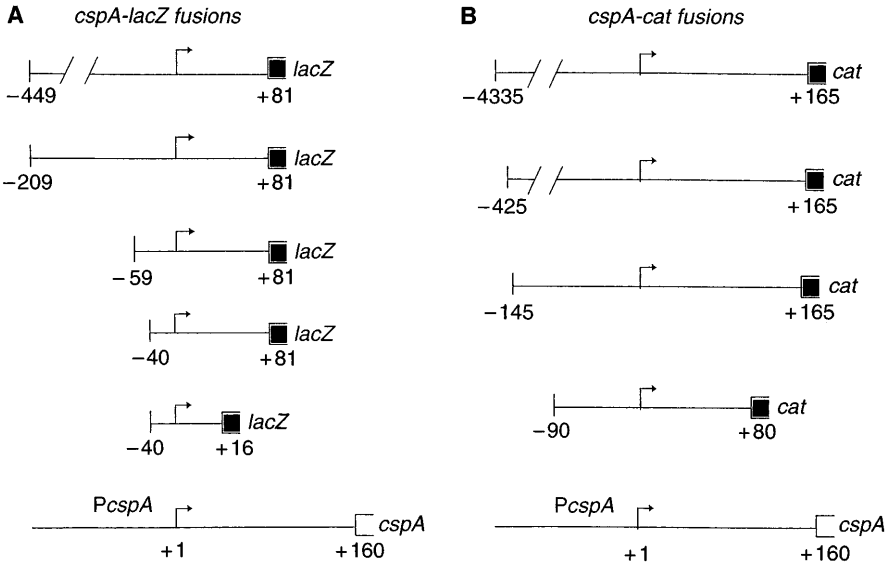


Fig. 1A, B Schematic representation of the *cspA-lacZ* and *cspA-cat* transcriptional fusions used in the present study. The diagram shows the five *cspA-lacZ* (**A**) and four *cspA-cat* (**B**) fusions constructed. A diagram of the chromosomal *cspA* gene with the translation start at +160 is shown below each set

seen that removal of the *cspA* promoter sequences upstream of –209 had no effect on the expression of *cspA-lacZ* at 37° C. A further deletion to –59 caused a 1.7-fold decrease in the β-galactosidase level, and removal of an additional 20 bp to –40 resulted in an additional twofold decrease. These reductions are probably due to the progressive loss of the AT-rich region present in this upstream segment of the *cspA* promoter; in fact, it is known that several strong promoters contain such so-called UP elements upstream of bp –40 and that their deletion can cause a substantial decrease in the promoter activity both in vivo and in vitro (Ross et al. 1993; Rao et al. 1994). Removal of the downstream sequence from +16 to +81, as in the P*cspA* –40/+16 construct, resulted in a twofold recovery of expression. With regard to the *cspA-cat* fusions, cells carrying constructs extending from +165 to –145, –425 and –4335 express essentially the same level of activity (control cells carrying pKK232-8 express virtually no CAT activity); this clearly indicates that the sequences that extend upstream from –145, up to –4335, have little or no effect on the levels of expression at 37° C. In contrast, cells carrying the –90/+80 fusion contained a substantially higher amount of *cat* transcript and expressed a substantially

Table 1 Activity of *cspA-lacZ* and *cspA-cat* operon fusions at 37° C

cspA promoter fragment in:		Reporter gene activity			
		lacZ		*cat*	
cspA-lacZ[a]	*cspA-cat*[a]	Transcript level[b]	Activity[c]	Transcript level[a]	Activity[d]
–449/+81		235	3620		
–209/+81		256	3820		
–59/+81		164	2240		
–40/+81		33	920		
–40/+16		137	1840		
p*lac*UV5		n.d.	4670		
	–4335/+165			82	11
	–425/+165			n.d.	13
	–145/+165			74	14
	–90/+80			475	77

[a] The *cspA* promoter fusions with *lacZ* and *cat* reporter genes are the same as those shown in Fig. 1
[b] The levels of *lacZ* and *cat* mRNA in the cells were determined by primer extension and Northern blotting analysis, respectively, as described in Materials and methods. The quantification was carried out in three separate experiments and the data presented in the Table are the results obtained in one of these experiments. The values represent arbitrary units derived from quantification by the Phosphoimager and therefore cannot be directly compared from one experiment to another. However, the relative amounts of the levels of transcript derived from the different experiments can be compared and proved to be reproducible within ±10%
[c] β-Galactosidase activity expressed as Miller Units was tested as described in Materials and methods. The values represent the average (±15%) of three separate experiments
[d] CAT activity expressed as percent of acetylated chloramphenicol was tested as described in Materials and methods. The values represent the average (±20%) of three separate experiments

higher level of CAT activity; in the experiment presented in Table 1, the degree of difference between this and the other constructs is approximately six-fold, but in other experiments, depending on the stage of growth, the difference was well over ten-fold.

The first important conclusion to be drawn from these experiments is that the activity of the promoter of the cold shock gene *cspA* is not restricted to low temperature, since reporter genes placed under its control can be actively transcribed at 37° C. This conclusion is also supported by in vitro transcription studies (not shown), which likewise indicate that the *cspA* promoter is efficiently utilized at 37° C. Our results also indicate that the upstream AT-rich region contained between positions –209 and –40 has a positive effect on transcription, while the region downstream of +16, and particularly that downstream of +80, is responsible for a reduction in the steady-state level of reporter transcripts and of the expression of the corresponding enzyme activities. This is likely to be due to the instability conferred on the transcript by the presence of the entire untranslated leader of *cspA*. In fact, the structure of this leader resembles a potential RNase E target (Cormack and Mackie 1992; Ehretsmann et al. 1992; Mackie 1992; Fang et al. 1997) and its disruption, as in the –209/+81 *cspA-lacZ* fusion and the –90/+80 *cspA-cat* fusion, drastically increases the half-life and the steady-state levels of the transcripts at 37° C (Goldenberg et al. 1996; Brandi et al. 1997).

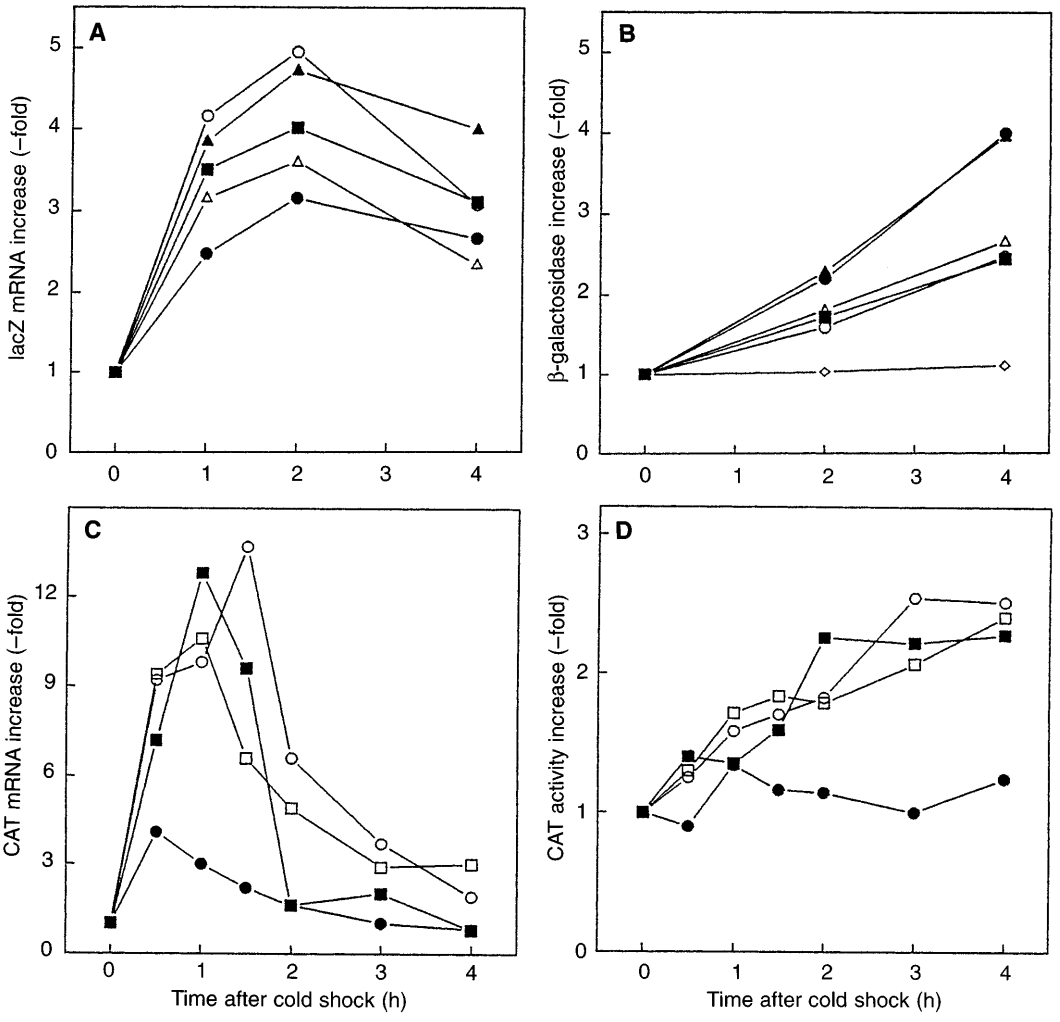
Activity of P*cspA* fusions during cold shock

To study the cold shock response of the *cspA* promoter, cells containing the above constructs were transferred in the exponential growth from 37° C to 15° C. At various intervals up to 4 h following the temperature shift, during which time there was only a marginal increase in cell mass, aliquots of the cultures were withdrawn for determination of the levels of *lacZ* mRNA (Fig. 2A) and β-galactosidase activity (Fig. 2B) or the levels of *cat* mRNA (Fig. 2C) and CAT activity (Fig. 2D).

The steady-state levels of *lacZ* mRNA increased in a similar manner with all constructs containing the *cspA* core promoter, the maximum increase (3- to 4.5-fold) being reached after approximately 2 h of cold shock. The maximal levels of cold shock induction were seen with the –40/+81 construct, which had the lowest activity at 37° C, and with the –209/+81 construct (Fig. 2A). The β-galactosidase activity expressed from the same constructs was found to increase by between 2.5-fold (–449/+81, –209/+81 and –40/+16) and 4-fold (–40/+81 and –59/+81) after 4 h at the low temperature. Under the same conditions, the control, consisting of the synthetic 40-bp *lac*UV5 promoter (–37 to +3) fused to *lacZ*, proved to be insensitive to the temperature change. Results similar to those presented in Fig. 2A and B were obtained in both IHF^+ and IHF^- backgrounds, as well as in cells cold shocked at higher cell density. These results seem to rule out an involvement of the stationary phase σ^S factor of RNA polymerase in determining the transcriptional activations observed in Fig. 2A. The levels of CAT mRNA showed a substantial increase between 1 and 2 h after imposition of cold shock; this increase was sharp and quantitatively similar for all constructs, with the exception of the shorter –90/+80 *cspA-cat* fusion, which showed a very modest increase over its already high level of expression at 37° C (Fig. 2C). The CAT activity expressed by the same cells was found to increase by approximately 2.5-fold after 4 h of cold shock for all *cspA-cat* promoter fusions, except for the –90/+80 *cspA-cat* construct already mentioned (Fig. 2D).

During the cloning of the *cspA* promoter, we encountered a spontaneous T:A to C:G mutation at –36 (see Fig. 3) in the –209/+81 *lacZ* fusion. This mutation caused a twofold decrease in expression at 37° C and an approximately 1.5-fold increase in the expression at 15° C, resulting in an overall threefold increase in the "cold shock response" both at the mRNA level assayed 1 h after imposition of cold shock, and the level of β-galactosidase activity assayed after 4 h of cold shock (not shown).

Taken together, the findings with both sets of P*cspA* fusions suggest that the core promoter (–40 to +16) of *cspA* is amenable to stimulation by cold shock, while the upstream sequences affect promoter expression at 37° C, but have no effect on the cold shock response. Interestingly, removal of the 20 bp between –40 and –60, which resulted in an over twofold decrease in promoter expression at 37° C, had no influence on cold shock activation, in spite of the fact that this region contains an AT-rich box (see Fig. 3) that is fairly well conserved in all bona fide cold shock *csp* genes (*cspA*, *cspB*, *cspG*) (Nakashima et al. 1996) and may constitute a UP-like element (Ross et al. 1993). In contrast to the upstream regions, the downstream sequences appear to reduce the expression of the constructs at 37° C, thereby amplifying the magnitude of induction following cold shock. These effects can be attributed to a negative influence of the +81 to +165 sequence on mRNA stability. This sequence corresponds to a long untranslated leader present in *cspA* mRNA (Fang et al. 1997), a feature shared also by *cspB* and *cspG* (Etchegaray et al. 1996; Nakashima et al. 1996). Whenever it was present in the fusions, this sequence was associated with a decrease in the half-life of the transcript at 37° C and towards the end of the cold shock lag (Goldenberg et al. 1996; Brandi et al. 1997). The cold inducibility of the *cspA* core promoter is reminiscent of the behavior of the *rrnB* P1 core promoter (–41 to +1), which retains the stringent response and can be shut off following amino acid starvation (Josaitis et al. 1995). One common characteristic shared by all cold-inducible promoters is that both the –35 and –10 elements deviate considerably from the consensus sequences recognized by the vegetative σ^{70} subunit of RNA polymerase. The –10 sequence of the *cspA* promoter is ctTAAT and the -35 motif is TTGcat, where the



lower case letters represent sites that deviate from the promoter consensus sequence. This property suggests that the interaction of RNA polymerase with the *cspA* promoter might be rate limiting at elevated, but not at low, temperatures and that some kinetic property of the promoter (i.e. the ease of promoter clearance) might allow its efficient use during cold shock. The increase in cold shock inducibility found following the T → C mutation at –36, which represents a further deviation from the –35 consensus, seems to support this premiss.

In addition to the non-canonical –35 and –10 hexamers, the *cspA* core promoter contains two peculiar sequences: the GCACATCA sequence and the CCAAT box. The first sequence, found between –6 and +1 in *cspA* (Fig. 3), is partially conserved in the *cspB* and *cspG* genes and is identical to a segment of DNA between –8 and –1 of phage λ P_L promoter which like the *cspA* promoter, can be activated by cold(Giladi et al. 1995). Since it has been found that upon mutation of this octamer there is a significant reduction in the degree of activation of the λ P_L promoter by cold shock in vivo (Giladi et al. 1995), we tested the significance of this sequence for *cspA* promoter function. A *cspA-lacZ*

Fig. 2A–D Cold shock expression of *lacZ* and *cat* reporter genes fused to various fragments derived from the *E. coli cspA* promoter. The *E. coli* cells carrying the indicated *cspA* fusions were grown at 37° C in LB to an A_{620} value of 0.4–0.5 for the *lacZ* fusions and of 1 for the *cat* fusions, before being shifted to 15° C. Aliquots were withdrawn at the indicated times and processed as indicated below. All data were normalized with reference to the levels of transcripts or of activity of the reporter genes found at 37° C just before the imposition of the cold shock. The actual levels at 0 time are given in Table 1. **A** Variations in the steady-state level of *lacZ* mRNA during cold shock. The mRNA levels in *E. coli* A6826 cells carrying the *cspA-lacZ* fusions in the chromosome as single-copy prophages were determined by primer extension analysis. The cells carried the following *cspA-lacZ* fusions: –449/+81 (*filled squares*), –209/+81 (*open circles*), –59/+81 (*filled circles*), –40/+81 (*filled triangles*), –40/+16 (*open triangles*). **B** β-Galactosidase activity expressed during cold shock. The β-galactosidase assays were carried out on cells processed as described (Goldenberg et al. 1996), according to the standard protocol (Miller 1972). The symbols are as in **A** with the addition of p*lac*UV5 (open diamonds). **C** Variations in the steady-state level of *cat* mRNA during cold shock. The *cat* mRNA level was determined by Northern blotting of total RNA prepared from *E. coli* DH5 cells harvested at the indicated times. The cells carried the following plasmids: –4335/+165 (*open squares*), –425/+165 (*filled squares*), –145/+165 (*open circles*), –90/+80 (*filled circles*). **D** Chloramphenicol acetyl-transferase (CAT) activity expressed during cold shock. The symbols are as in **C**

Fig. 3 Sequence of the *E. coli cspA* promoter region from –59 to +16. The sequence is taken from Tanabe et al. (1992). The transcription start site (+1) is depicted by an *arrow*, the *horizontal bars* indicate the –10 and –35 regions, and the *numbered vertical lines* the boundaries of the cloned minimal *cspA* promoter fragment –40/+16 and the AT-rich sequence between –40 and –59. The CCAAT and GCACATCA sequences that were mutagenized to GGAAT and CGAGATCA, respectively, and the –36 site where the T:A→C:G spontaneous mutation occurred are *boxed*

fusion (–209/+81) mutated, at three nucleotides in this site, to CGAGATCA was transferred to the λB299 phage and introduced into A6826 cells as a prophage. No significant differences were found between the mutated and wild-type promoters during cold shock as indicated by either β-galactosidase activity or mRNA levels (not shown).

The CCAAT (Y-box) sequence present between the –35 and the –10 elements (Fig. 3) may be involved in an autoregulatory loop controlling *cspA* expression, since CspA, which is a Y-box binding protein (Wolffe et al. 1992), has been shown to stimulate transcription of at least two cold shock genes, *hns* and *gyrA*, through the recognition of this sequence motif (La Teana et al. 1991; Jones et al. 1992; Brandi et al. 1994). To test this hypothesis, a possible interaction of CspA with this CCAAT box was investigated by various approaches. First, we looked at the accessibility to the restriction endonuclease *Pfl*MI of the CCA(N5)TGG sequence, which partially overlaps the CCAAT element (Fig. 3). We found that cleavage of a purified *cspA* promoter fragment (250 bp) by *Pfl*MI was unaffected by the presence of up to 56 μM purified CspA protein (not shown). Secondly, in vitro transcription of the –425/+165 *cspA-cat* construct carried out in the presence and absence of purified CspA showed only a marginal stimulation (approximately 60%) at 3.5 μM of CspA (not shown). Finally, the CCAAT site was mutated to GGAAT in three of the *cspA-lacZ* fusions described above and these mutated promoters were cloned upstream of the *lacZ* gene in the transcriptional vector pIK86. The resulting plasmids were then introduced into an A6826 derivative carrying the *pcnB* mutation which greatly reduces plasmid copy number (Liu and Parkinson 1989). The results presented in Table 2 clearly show that the expression of β-galactosidase from the mutant *cspA* promoters was not significantly affected either at 37° C or following cold shock. Similar results were also obtained when the mutated –59/+81 *cspA-lacZ* fusion was introduced as a single-copy λ prophage into A6826 cells (data not shown).

Thus, neither our in vivo nor in vitro results give any indication for the participation in the regulation of *cspA* promoter expression of the two peculiar elements present within the cold-inducible 56-bp (–40/+16) core promoter region of *cspA* (the GCACATCA and the CCAAT sequences). It is possible, however, that this negative finding might be due to the inability of our experimental tests to detect an activity which might occur only under special conditions.

Evidence for post-transcriptional control of the cold-shock response

The increase of the cellular levels of the various transcripts expressed from the various P*cspA* fusions (Fig. 2A, C) and the increase in the activities of the corresponding reporter gene products (Fig. 2B, D)

Table 2 Activity of plasmid-borne *cspA-lacZ* fusions carrying CCAAT → GGAAT mutations in the *cspA* promoter

CCAAT site	*cspA* promoter fragment[a]	β-Galactosidase (Miller units)			mRNA level[c]
		37° C	15° C[b]	15° C/37° C	15° C/37° C
Wild type	–209/+81	3380	9540	2.8	nt
mutant	–209/+81	2760	9520	3.4	nt
Wild type	–59/+81	3320	10060	3.0	6.0
mutant	–59/+81	4950	10780	2.2	4.0
Wild type	–40/+16	1220	3070	2.5	3.3
Mutant	–40/+16	1100	2740	2.5	4.5
None[d]	none[d]	18	16	–	–

[a] The indicated *cspA-lacZ* transcriptional fusions were cloned into pIK86 and transformed into *E. coli* A7873 cells (a *pcnB*::Tn*10* derivative of strain A6826). Overnight cultures were diluted 1:100 and grown at 37° C in LB medium containing 40 μg/ml kanamycin and the temperature was shifted to 15° C at an OD_{600} value of 0.4–0.6. The β-galactosidase activity and the level of *lacZ* mRNA were determined as described in Materials and methods
[b] After 4 h at 15° C
[c] After 1 h at 15° C
[d] Control pIK86 without *lacZ* promoter

during cold-shock display important parallels with, as well as important differences from, the behaviour of the cold-shock gene *cspA* under the same conditions. In fact, inspection of the results obtained with the two *cspA* promoter fusions reveals the interesting fact that, while the levels of the transcripts reach their maxima and then decline (more or less drastically) towards the end of cold shock, the products of the two reporter genes continue to accumulate even when the levels of their mRNAs are substantially reduced. With respect to the RNA levels, these results are similar to those normally obtained for the expression of chromosomal *cspA*, since in this case the steady-state level of the mRNA reaches its maximum between 1 and 2 h after imposition of cold shock and then progressively declines toward the initial value (not shown); with regard to translation, however, the situation is different since, unlike the case of the reporter genes, maximal translation of CspA coincides with the time at which the concentration of the mRNA reaches its peak.

That the translation of the proteins encoded by the two reporter genes is delayed relative to the translation of CspA and other cold shock proteins can be seen in the following experiments. When cells harbouring the *cspA-cat* fusion are grown in minimal medium and then subjected to cold shock and pulse labelled with [^{35}S]methionine at different time points, the maximum incorporation of radioactivity into chloramphenicol acetyltransferase (CAT) occurs after the maximal rate of incorporation into CspA has already taken place, as can clearly be seen in the autoradiogram presented in Fig. 4A and from its quantitative analysis (Fig. 4B). As is also seen in Fig. 4, the behaviour of two other cold shock proteins, namely the DEAD-box helicase CsdA (Iost and Dreyfus 1994; Jones et al. 1996) and translation initiation factor IF2, is similar to that of CspA and different from that of CAT. It can be concluded, therefore, that while the synthesis of *bona fide* cold shock proteins increases immediately at the onset of cold shock, reaches its maximum about 2 h later and then declines, the translation of a non-cold shock protein such as CAT continues to increase through the later stages of cold shock, reaching its maximum towards the end of the cold shock lag. Thus, while the maximum level of translation of the cold shock proteins occurs at a time when the steady-state level of the respective mRNA has reached its peak, the highest rate of synthesis of the non-cold shock protein CAT occurs when the intracellular level of its mRNA has already substantially declined. This cold-induced translational selectivity is clearly evident in the plot presented in Fig. 5 in which

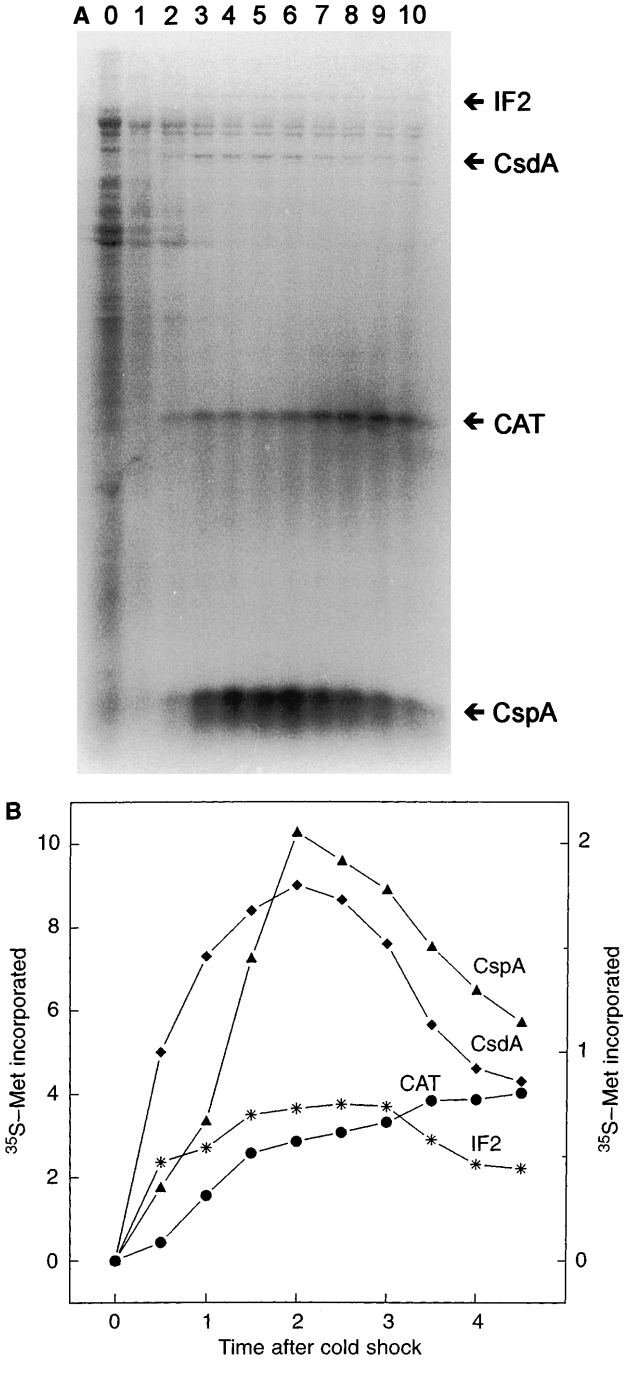


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Fig. 4A, B In vivo synthesis of cold shock and non-cold shock proteins at various times after temperature shift. *E. coli* DH5 cells carrying the –425/+165 *cspA-cat* fusion were grown at 37° C in M9 minimal medium supplemented with yeast extract (0.01%), all amino acids except methionine, and ampicillin (60 mg/ml). When the culture reached an A_{500} value of 1.0, a 1-ml aliquot was pulse labelled for 5 min at 37° C with [^{35}S] methionine (7 mCi; 3000 Ci/mMol). The rest of the culture was transferred to 10° C and 1-ml aliquots of the culture were pulse labelled (30 min at 10° C) with 7 mCi of [^{35}S] methionine (3000 Ci/mMol) at consecutive intervals of 30 min. The amount of radioactivity associated with individual proteins was determined after SDS-PAGE of total cell extracts followed by autoradiography of the dried gel; the autoradiogram was scanned in a Molecular Imager (Biorad). **A** Autoradiogram of the SDS-polyacrylamide gel. Lane 0, cells pulse labelled at 37° C prior to cold shock; lanes 1–10, cells pulse labelled for ten consecutive intervals of 30 min each at 10° C. **B** [^{35}S]Methionine incorporated before and after cold shock. The axis on the right refers to CspA (*filled triangles*) and CAT (*filled circles*), whereas the axis on the left is for CsdA (*filled diamonds*) and IF2 (*asterisks*)

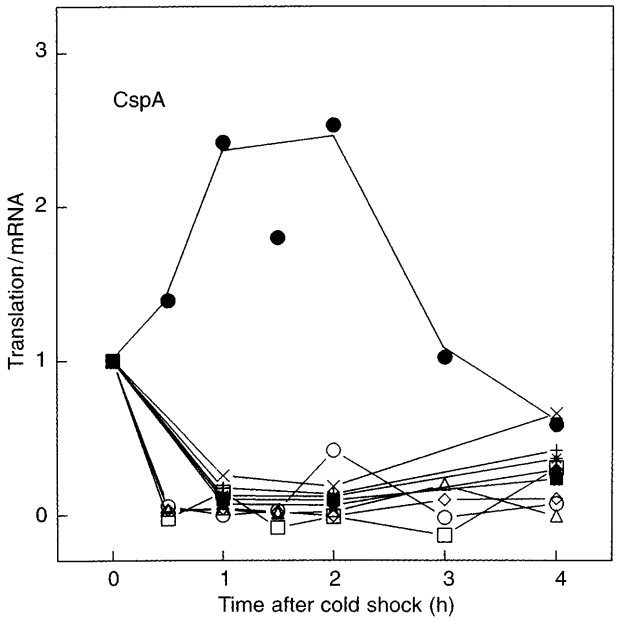

Fig. 5 Efficiency of translation of *cspA*, *lacZ* and *cat* mRNAs during cold shock. The translational efficiency of each mRNA was determined from the accumulation of each protein product within each time interval. Translational efficiency is represented as the ratio between the steady-state level of each mRNA determined at each time after cold shock and the amount of the corresponding protein synthesized during the corresponding period. CspA synthesized/*cspA* mRNA level (*filled circles*), CAT synthesized/mRNA level for the *cat* transcripts originating from the following P*cspA* fusions: –90/+80 (*open squares*), –145/+165 (*open triangles*), –425/+165 (*open circles*), –4335/+165 (*open diamonds*) and β-galactosidase synthesized/mRNA level for the *lacZ* transcripts originating from the following P*cspA* fusions: –40/+16 (*asterisks*), –40/+81 (+), –59/+81 (x), –209/+81 (*filled diamonds*) and –449/+81 (*filled squares*)

Table 1 Activity of *cspA-lacZ* and *cspA-cat* operon fusions at 37° C

Table 2 Activity of plasmid-borne *cspA-lacZ* fusions carrying CCAAT → GGAAT mutations in the *cspA* promoter

the levels of newly synthesized CspA and of the proteins encoded by the reporter genes in each given time interval after cold shock are normalized with respect to the corresponding levels of their mRNAs and plotted as a function of time after imposition of cold shock. As seen from this Figure, at the onset of the global response, the protein biosynthetic apparatus of the cell is very efficient at translating the "cold-shock" *cspA* mRNA, and rather inefficient in translating a "normal" mRNA, such as *cat* or *lacZ* mRNA; as the growth lag caused by the cold shock approaches its end and the cells start growing again, however, this situation begins to reverse.

Conclusions

In this paper we have established that the core promoter of *cspA* can be induced by cold shock. Since the magnitude of the enhancement detected in our in vivo and in vitro experiments is quantitatively modest, we suggest that this transcriptional enhancement could significantly contribute to cold shock induction of this gene only if coupled to post-transcriptional controls, such as the reported alterations in mRNA stability and in the translational apparatus (Goldenberg et al. 1996; Brandi et al. 1996, 1997). With regard to the latter point, it should benoted that the results presented here (Figs. 2, 4 and 5) are in full agreement with the hypothesis that the ribosome might be the "sensor" for both cold- and heat-shock responses (VanBogelen and Neidhardt 1990) and with the previous findings that the translational machinery of cold-shocked cells is modified in such a way as to translate *cspA* mRNA more efficiently (Brandi et al. 1996). In view of the reported changes in *cspA* mRNA stability after cold shock (Goldenberg et al. 1996; Brandi et al. 1996), it should be recalled that the half-life of the *cspA* mRNA undergoes a very dramatic decrease with time of cold shock, while the half-life of the transcript from the –209/+81 *cspA-lacZ* fusion remains fairly constant (Goldenberg et al. 1996), suggesting that the 5′UTR of this mRNA plays an important role in determining its stability, a conclusion reached also by Fang et al. (1997) and Brandi et al. (1997). The present data provide further support for this idea, since it has been shown (Fig. 2C) that the steady-state levels of the transcripts derived from the *cspA-cat* fusions (with the exception of the –90/+80 construct) decline rapidly towards the end of cold shock, thus displaying a somewhat different behaviour from that of the transcripts of the *cspA-lacZ* fusions whose levels decline only very slightly (Fig 2A). In light of what has been mentioned above, this difference in stability can be attributed to the presence (in all the *cspA-cat* fusions except the –90/+80 construct) or absence (in the *cspA-lacZ* fusions) of the segment spanning the region from +81 to +165.

Finally, a possible role of the CsdA RNA helicase in mediating the stabilization of the CspA mRNA (Brandi et al. 1997) is suggested by the finding that the synthesis of this protein seems to precede that of CspA (Fig. 4B).

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