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Interaction of the main cold shock protein CS7.4 (CspA) of *Escherichia coli* with the promoter region of *hns*

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Summary — *Escherichia coli* protein CS7.4 (CspA), homologous to the class of eukaryotic Y-box DNA-binding proteins, is a cold shock transcriptional activator of at least two genes, *hns* and *gyrA*. It was demonstrated that all or nearly all the elements necessary for the stimulation of *hns* transcription by CS7.4 protein are located in the proximal 110 bp DNA fragment of this gene with no additional elements being present in a longer fragment (660 bp) extending further upstream from the *hns* promoter. Protein CS7.4 bound strongly to the 110 bp segment of the *hns* promoter in crude extracts of cold shocked cells, but the purified protein displayed a weak interaction with the same DNA fragment. Purified CS7.4 protein also caused increased or decreased accessibility to DNase I at different sites of the 110 bp fragment of *hns* but the majority of these effects was seen only in the presence of RNA polymerase. Since gel shift experiments showed that protein CS7.4 stimulated the binding of RNA polymerase to the promoter of *hns* and since it is known that there are similarities between CS7.4 and ssDNA-binding proteins, we suggest that formation of the open complex by the RNA polymerase or protein-protein contacts between CS7.4 and the RNA polymerase are prerequisites for and/or the effects of the interaction of CS7.4 with its DNA target. The presence of a conserved CCAAT element in the *hns* promoter region, on the other hand, was found not to be stringently required for cold shock activation since expression in *E coli* of an *hns-cat* fusion containing the *Proteus vulgaris hns* promoter lacking a CCAAT box increased over four-fold after cold shock.

bacterial nucleoid / *hns* promoter / cold shock protein / protein-DNA interaction

Introduction

When the temperature of exponentially growing cultures of *E coli* is lowered from 37°C to 10–15°C, the expression of a set of approximately 13 genes is increased two- to ten-fold while that of another gene, *cspA*, is increased over 100-fold [1, 2]. The list of the cold shock genes identified so far is presented in table I. Among the cold shock genes, *cspA*, which encodes CS7.4, a protein of 69 amino acids rich in charged and aromatic residues [2], plays a particularly important role. A protein homologous to *E coli* CS7.4 has also been found in *Bacillus subtilis*. This protein, designated CspB, is 61% identical to its *E coli* counterpart and was shown to play a role in preserving cell viability after cold shock [3]. The three-dimensional structure of this protein has recently been elucidated by X-ray crystallography [4] and NMR spectroscopy [5]; these studies revealed that the protein consists of five anti-parallel beta-strands divided into two subdomains, one involved in protein-protein interaction, the other in nucleic acid binding. One of the functions of CS7.4 is to act as cold shock transcriptional enhancer of at least two cold shock genes, *hns*, encoding the major nucleoid protein H-NS [6] and *gyrA*, encoding the A subunit of DNA gyrase [7]. The Csp proteins are sequence-related to one of the domains of eukaryotic Y-box factors [8], a family of nucleic acid-binding proteins which includes human YB-1 [9], DbpA and DbpB [10] and *Xenopus* FRG Y1 and FRG Y2 [11]. The Y-box factors recognize a CCAAT sequence motif, the same present two and three times in the promoter regions of *hns* and *gyrA*, respectively. The same conserved CCAAT sequence is also present (twice) in *cspA* itself as well as in the cold-inducible promoters of two mutants designated WQ3 and WQ11 [12].

As mentioned above, the CS7.4 protein has been identified as the factor present in extracts of cold shock cells responsible for the transcriptional enhancement of *hns* recognizing a 110 bp DNA fragment (fig 1) containing the –10 and –35 elements of the promoter and a CCAAT motif located between the transcriptional startpoint and the first few codons of H-NS [6].

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Table I. Cold shock genes and products from *Escherichia coli.*

Gene	*Map position*	*Gene product*
recA	58'	RecA
nusA	69'	NusA
infB	69'	IF2 ($\alpha + \beta$)
pnp	69'	Polynucleotide phosphorylase
aceE	3'	Pyruvate dehydrogenase (lipomide)
aceF	3'	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase)
cspA	79'	CS7.4
hns	27'	H-NS
gyrA	48'	DNA gyrase (subunit A)

In the present paper we investigated whether additional signals for CS7.4 might exist upstream of its 110 bp target and determined the effects of CS7.4 on the accessibility of this DNA fragment to DNase I. Finally, to determine the relevance of a conserved CCAAT motif in eliciting the cold shock enhancement we investigated the cold inducibility of the *hns* promoter derived from *Proteus vulgaris* which, unique among the known Enterobacteriaceae *hns* genes (fig 1), lacks this motif. Our results indicate that no additional cold shock signal is present in the *hns* promoter, at least within 550 bp upstream the 110 bp fragment constituting the main target and that cold shock induction *in vivo* can occur also in the absence of a CCAAT sequence. We also show that binding of purified CS7.4 to the 110 bp target is weak in the absence of other proteins (probably the RNA polymerase) and that, in the absence of RNA polymerase, CS7.4 has only marginal effects on the accessibility of DNase I to the 110 bp fragment. Based on our results, we suggest a possible mechanism of transcriptional enhancement by this protein.

Materials and methods

The DNA manipulations for the construction of pKK110 and pKK660 carrying a promoter-less *cat* gene fused to segments of the promoter region of *E coli hns* consisting of 110 and 660 bp, respectively have been described previously [6, 16]. Two independent preparations (prep A and B) of CS7.4 purified as described [6] were used in the present study. Expression of CAT activity *in vitro* by transcription-translation systems programmed with either pKK110 or pKK660 [6], electrophoretic mobility shifts [6], affinity chromatography of ^{35}S-labelled protein on matrix-bound DNA [6] and DNase I footprinting [16] were carried out essentially as described. Further details are given in the appropriate figure legends.

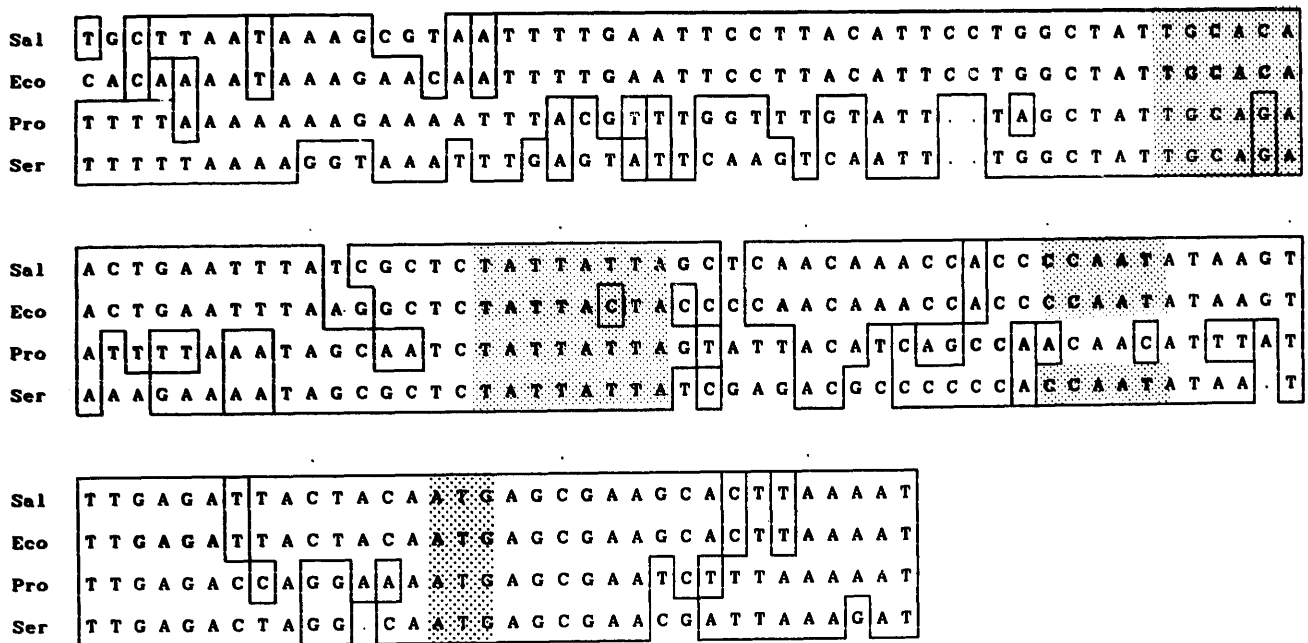

Fig 1. Comparison of the DNA sequences around the promoter regions of *hns* from four Enterobacteriaceae. (Sal, *Salmonella typhimurium*; Eco, *Escherichia coli*; Pro, *Proteus vulgaris*; Ser, *Serratia marcescens*). The sequences are aligned from the *E coli* ATG initiation codon of H-NS (stippled). Also stippled are the −10 and −35 promoter elements identified in *E coli* [13] and, when present, the CCAAT cold shock box. Identical nucleotides are boxed. The sequences are taken from [13, 14]. A conserved CCAAT has also been found in the *hns* promoter region of *Shigella flexneri* whose sequence [15] is not shown in the figure.

Results

Cold shock enhancement of transcription from the *hns* promoter can be seen *in vitro* by monitoring CAT activity produced by cell-free transcription-translation systems programmed with *hns-cat* fusions using extracts from cold shocked cells [6]. In fact, due to the presence of two translational stop codons preceding the CAT coding sequence, this assay measures effects on the transcriptional activity of the template [6]. As seen in figure 2, the systems programmed with pKK660 were more active than those programmed with pKK110. This difference is probably due to the stimulation of transcription of pKK660 by Fis protein, since this protein was found to have several specific binding sites in the promoter region of *hns*, most of them localized upstream of the *Eco*RI site which is the upstream limit of the 110 bp fragment and to stimulate transcription from the *hns* promoter ([16] and Falconi *et al*, manuscript in preparation). Compared to the extract of control cells grown at 37°C, the extract of cells subjected to 2 h cold shock produced a higher amount of CAT activity when programmed with either pKK110 or pKK660 and the extent of the cold shock stimulation was the same (approximately two-fold) with both templates (fig 2). This indicates that all or nearly all signals required for cold shock enhancement are present in the 110 bp fragment of *hns* promoter.

In the next experiment (fig 3), the effects of post-ribosomal supernatants from control and cold shocked cells on the electrophoretic mobility of the 110 bp (panel A) and 660 bp (panel B) fragments of *hns* promoter were compared. To avoid gel shift effects due to the interaction of the DNA fragments with the main cellular DNA binding proteins (*eg* HU and H-NS), the supernatants had been previously deprived of these proteins by passage through DNA cellulose. As seen from the figure, the post-ribosomal proteins derived from cells harvested after 3 h cold shock (even-numbered lanes) caused the most dramatic effect on the electrophoretic mobility of both fragments which were shifted with nearly the same efficiency. By contrast, the control cell extract (odd-numbered lanes) produced a less pronounced effect on the 660 bp fragment and a negligible effect on the 110 bp fragment. It is noteworthy that the gel shifts caused by the extracts from the control and cold shocked cells are not only quantitatively but also qualitatively different. This finding, which is particularly evident with the 660 bp fragment at lower inputs of protein, indicates that different proteins in the extracts were responsible for the observed gel shifts. Protein CS7.4, present in the extract from cold shocked cells, recognized and bound with the same efficiency to the 110 and 660 bp fragments, possibly helped by another factor (see below). On the other hand, for the reasons given above, the

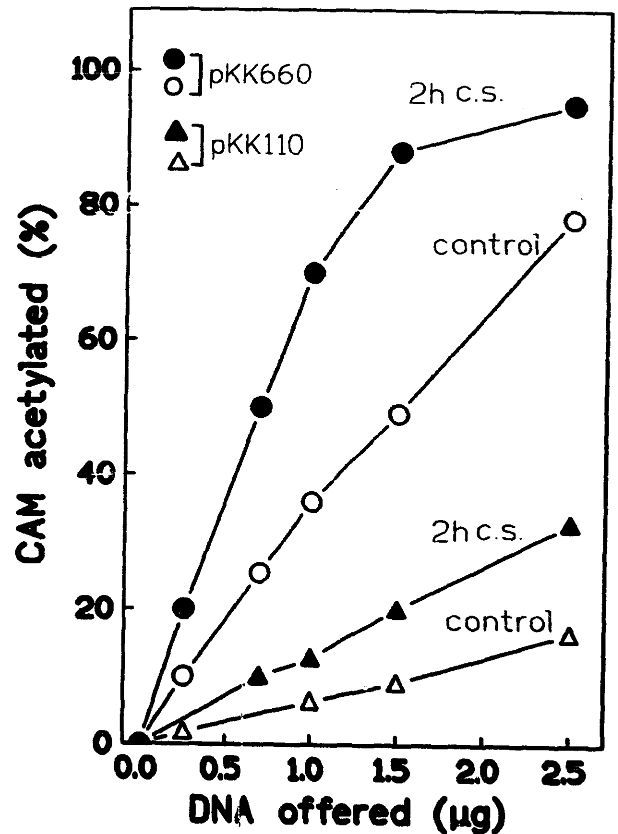

Fig 2. CAT activity expressed *in vitro* from *hns-cat* fusions in coupled transcription-translation systems prepared with extracts from control cells growing at 37°C (open symbols) and cells after 2 h of cold shock (closed symbols) as a function of increasing amounts of template pKK110 (△, ▲) or pKK660 (●, ○).

protein from control cells which affected the mobility of the 660 bp fragment much more than that of the 110 bp fragment is likely to be Fis, probably in combination with RNA polymerase.

Among the large number of *in vivo* ^{35}S-labelled proteins present in the post-ribosomal supernatant of cold shocked cells after removal of the major DNA-binding proteins, only CS7.4 was retained by matrix-bound 110 bp (fig 4A) and 660 bp (fig 4B) fragments of *hns*; the fact that CS7.4 was eluted from the two DNA fragments at approximately the same ionic strength is a further indication that this protein has similar affinity for the 110 and 660 bp fragments of *hns*. The cold shock specificity of this interaction was demonstrated by the finding that neither DNA frag-

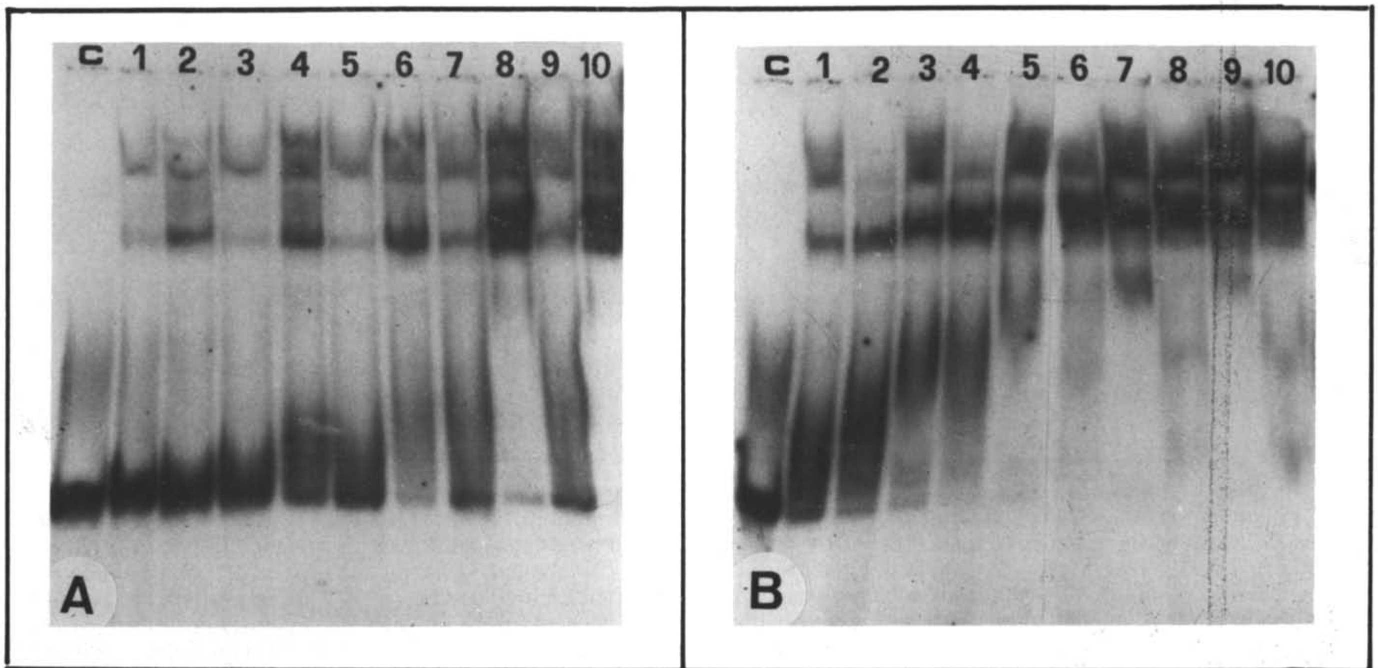

Fig 3. Electrophoretic mobility shift of the *hns* 110 bp (**A**) and 660 bp (**B**) promoter region by increasing amounts of extracts from control and cold shocked (3 h at 10°C) *E coli* cells. The reaction mixtures (15 μl) contained approximately 5 ng of ^{32}P-end-labelled DNA fragments derived from *hns* and 0.6 μg poly d(IC) as competitor DNA in 26 mM Tris-HCl (pH 7.7), 60 mM KCl, 40 mM NH_4Cl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.15 μg BSA. The control samples (lanes c) contained no other additions. The other samples contained increasing amounts of postribosomal supernatant (S100) fractions passed through calf thymus DNA-cellulose columns to remove the major DNA binding proteins obtained from cells growing at 37°C (odd-numbered lanes) or from cold shocked cells (even-numbered lanes). The total amount of protein present in each mixture was 2.2 μg (lanes 1 and 2), 4.4 μg (lanes 3 and 4), 6.5 μg (lanes 5 and 6), 8.6 μg (lanes 7 and 8), 10.8 μg (lanes 9 and 10). After 30 min incubation at 10°C, the samples were subjected to electrophoresis in 10% polyacrylamide gels in TBE and autoradiography.

ment retained protein from a comparable fraction of labelled proteins derived from control cells (not shown).

As seen in figure 5, purified CS7.4 protein altered the electrophoretic behavior of the 110 bp fragment of *hns* (*cf* lanes c and 1) to give rise to a band having the same mobility shift as that displayed by the same fragment (indicated by the lowest arrow) in the presence of the crude post-ribosomal supernatant of cold shocked cells (lane 6). The affinity of the purified CS7.4 protein for its DNA target seems to be much lower than expected from the results obtained in the presence of crude extracts, however (figs 3, 4). In fact, unlike the crude extract which caused the complete disappearance of the original non-retarded band, purified CS7.4 protein caused the shift of only a fraction of the 110 bp fragment while a large proportion of the fragment still migrated to its normal position; increasing amounts of purified protein (lanes 1–5) did not cause any substantial increase in the proportion of the retarded fragment. In addition, in contrast to the crude extract which also produced larger retardation effects (indicated by the two upper arrows), increasing amounts of CS7.4 did not cause a further retardation of the DNA fragment. Finally, it should be noted that addition of purified CS7.4 protein to the crude extract of cold shocked cells did not produce any additional retardation effects (*cf* lanes 6 and 7). Taken together, these results and those of figures 3 and 4 suggest that the affinity of the purified CS7.4 protein for its DNA target is substantially increased by one or more additional factor(s) present in the crude extract and which is/are not ^{35}S-labelled during the pulse given in the experiment of figure 4. The presence of these factor(s) also allowed CS7.4 to cause the larger electrophoretic shifts observed in lanes 6 and 7 of figure 5. The data presented below strongly suggest that this factor might be the RNA polymerase.

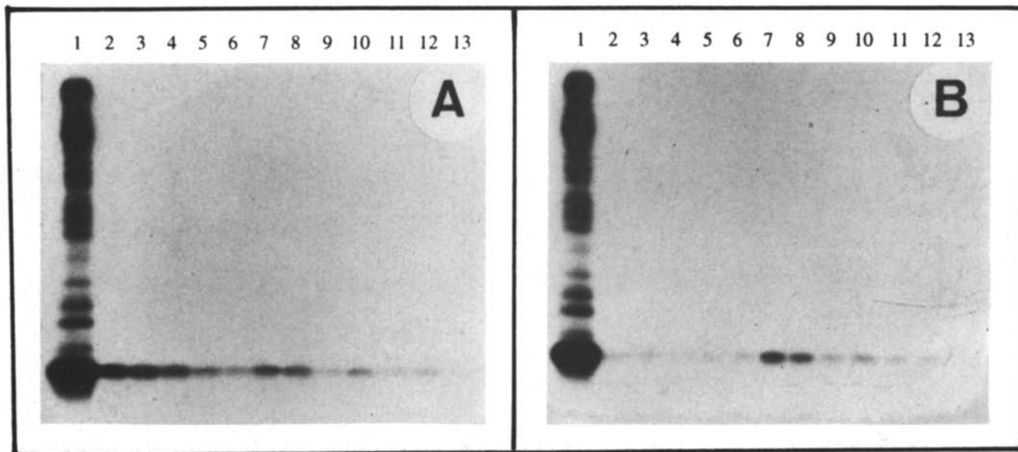

Fig 4. Fractionation of ^{35}S-labelled proteins by affinity binding to immobilized *hns* 110-bp (A) or 660-bp (B) promoter regions. Each incubation mixture contained the DNA fragments immobilized on MagniProbe resuspended in 540 µl of 20 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 20 mM NaCl and 260 µl of *in vivo* ^{35}S-labelled S100 fractions (freed of DNA-binding proteins by passage through DNA cellulose) prepared from *E coli* cells after 1.5 h cold shock at 10°C. The protein was bound to the immobilized DNA and eluted by a series of washes with increasing concentrations of NaCl as previously described [6]. Aliquots of each wash were analyzed by SDS/18% PAGE and autoradiography. Lanes: 1, the first supernatant (*ie* total unbound proteins); 2–4, three successive washes with 20 mM NaCl; 5–13, washes with 50 mM, 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 800 mM, and 1.0 M NaCl.

The experiments presented in figure 6 show the DNase I footprints generated by RNA polymerase and CS7.4 protein on the template (fig 6A) and non-template (fig 6B) strands of the 110 bp fragment of *hns*. As seen from the figure, unlike the RNA polymerase which caused extended effects on both fragments, the CS7.4 protein alone had rather marginal effects on the pattern of DNase I cleavage of either fragment. These effects are only of one type, namely the increased accessibility to the nuclease of a few sites indicated by closed triangles in figure 7. In the presence of RNA polymerase, however, increasing amounts of protein CS7.4 resulted in a large number of effects consisting either in an increased (black arrowheads) or a decreased (open arrowheads) accessibility of specific sites to DNase I. In some cases, the cold shock protein seemed to accentuate the effects of the RNA poly-

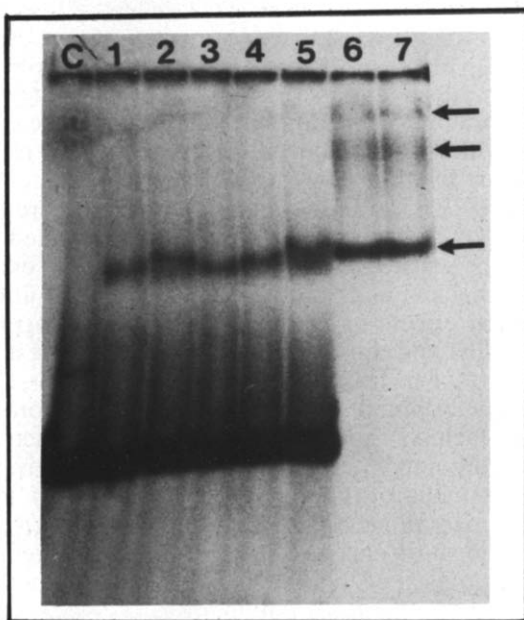

Fig 5. Electrophoretic mobility shift of the *hns* 110 bp promoter region by increasing amounts of purified CS7.4 protein. The reaction mixtures (15 µl) contained approximately 5 ng of ^{32}P-end-labelled 110 bp *Eco*RI- *Hind*III DNA fragment of *hns* and 10 ng poly (dI-dC) as competitor DNA in 20 mM Tris-HCl (pH 7.7), 0.1 mM EDTA, 1.6 mM DTT, 10% glycerol. The control sample (lane c) contained no other additions. The other samples contained CS7.4 in the following concentrations (µM): lane 1, 2.25 (prep A); lane 2, 4.5 (prep A); lane 3, 2.7 (prep B); lane 4, 5.4 (prep B); lane 5, 16.2 (prep B). The samples of lanes 6 (no CS7.4) and 7 (5.4 µM CS7.4, prep B) also contained 6 µg of total protein from the post-ribosomal supernatant (S100) fractions derived from 3 h cold shocked cells as described in the legend to figure 3. After 10 min incubation at 10°C, the samples were subjected to electrophoretic separation in 10% polyacrylamide gels in TBE and autoradiography.

merase; in other cases, however, the effects of CS7.4 were antagonistic to those of the RNA polymerase re-exposing to the cleavage some sites which had been protected by the polymerase alone. The latter finding and the specific albeit limited effects caused by CS7.4 in the absence of the polymerase indicate that the cold shock protein is capable of independent binding to the DNA. A summary of the CS7.4 protein effects on the 110 bp fragment of *hns* is presented in figure 7. Overall, DNase I digestion of the entire 110 bp fragment appears to be affected by CS7.4 in the presence of RNA polymerase with the effects being quite asymmetric on the two DNA filaments. The most extended effects are seen in the non-template strand and consist

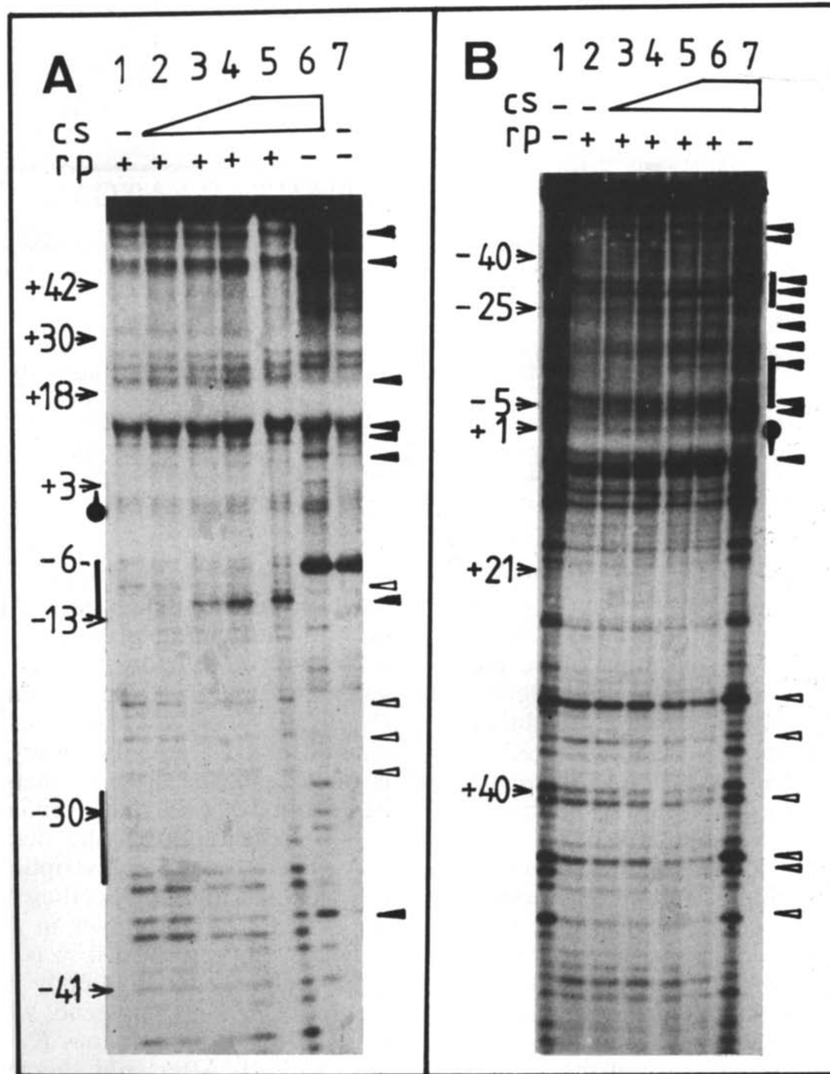

Fig 6. DNase I footprinting of the promoter region of *E coli hns* in the presence of CS7.4 and/or RNA polymerase. The reaction mixtures (20 μl) contained 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 2 mM DTT, 1 mM spermidine, 0.5 mM each CTP and ATP, 50 μg/ml BSA and an approximately 110-bp fragment excised from pKK110 by either *Eco*RI–*Pst*I (A) or *Eco*RI–*Hin*dIII (B) end-labelled with ^{32}P-dATP or ^{32}PdCTP by a fill-in reaction using the Klenow fragment of DNA polymerase. Furthermore, when specified, each reaction tube contained 0.4 units of RNA polymerase and the following concentrations (μM) of purified CS7.4. **A.** Lanes: 1–7 (0, 0, 4, 10, 15, 20, 20). **B.** Lanes 1–7 (0, 2, 6, 12, 18, 18, 0). After preincubation for 1 h at 10°C, 10 ng of DNase I were added to each sample and incubation continued for an additional 3 min at the same temperature before the electrophoretic analysis [16]. The vertical bars indicate the positions of the –35 and –10 elements of the promoter. The solid circle with arrowhead indicates the startpoint and the direction of transcription. The horizontal arrowheads indicate increased (closed symbol) or decreased (open symbol) accessibility to DNase I due to the presence of CS7.4.

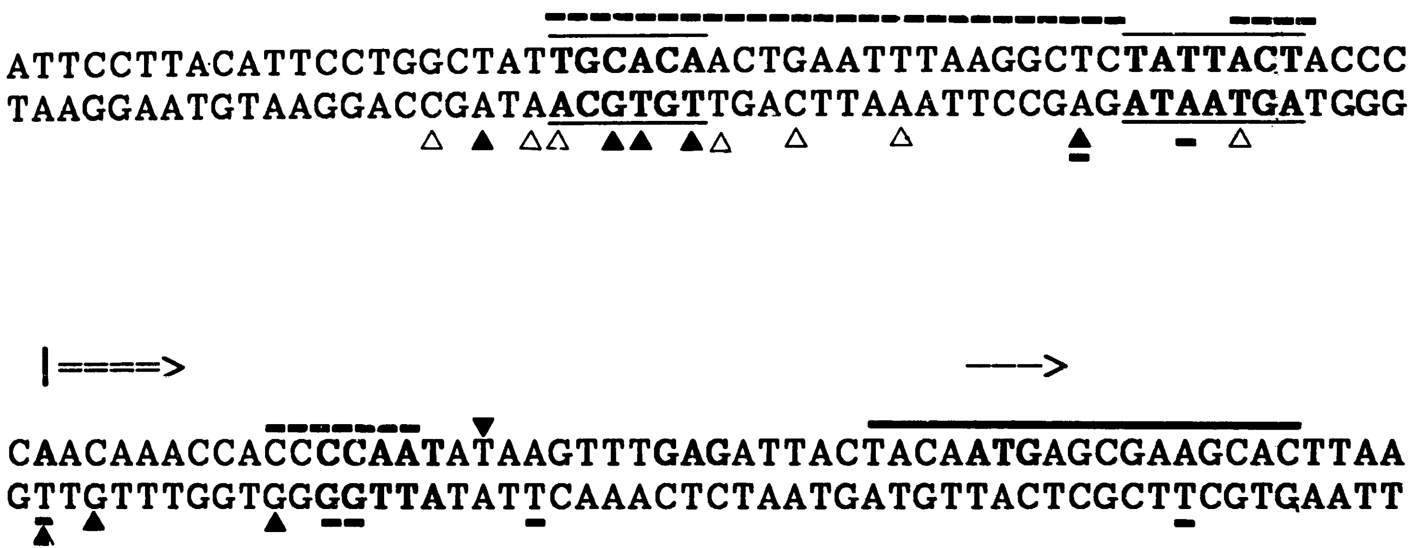

Fig 7. Sequence of the *Eco*RI–*Fok*I 110 bp DNA fragment from the proximal region of *hns* used to construct the *hns–cat* fusion in pKK110. The relevant sequences present in the fragment (from left to right: the –35 and –10 element of the promoter, the transcriptional startpoint, the CCAAT motif, the Shine-Dalgarno sequence, and the H-NS initiation triplet) are indicated by bold letters. The remaining symbols summarize the results of the effects of CS7.4 in four DNase I foot-printing experiments including those presented in figure 6A, B: the continuous and discontinuous lines indicate regions of overall decreased and increased accessibility, respectively, induced by CS7.4 in the presence of RNA polymerase. Sites of increased cleavage in the presence of CS7.4 alone (▲), sites of decreased accessibility by CS7.4 in the presence of RNA polymerase (△).

of an increased accessibility to DNase I in the central region of the fragment (*ie* the area between the –35 and the –10 elements of the promoter) as well as downstream of the transcription startpoint where the CCAAT cold shock box is located; in this region, CS7.4 protein caused primarily increased accessibility to the nuclease. Further downstream CS7.4 and RNA polymerase cause an extended protection of the same filament. The most important conclusion to be drawn from these experiments, however, remains the fact that protein CS7.4 affected the footprint of the promoter region of *hns* primarily in the presence of RNA polymerase as if only in its presence the cold shock protein could form a stable complex with DNA. Consistent and complementary with this notion is the finding that the cold shock protein stimulated the interaction of the RNA polymerase with the promoter region of *hns* as shown by the increased gel shift of the 110 bp *hns* fragment produced by increasing amounts of RNA polymerase in the presence of CS7.4 protein (fig 8).

The *hns* promoter region of *P vulgaris* is unique among the corresponding Enterobacteriaceae sequences known sofar for the lack of a conserved CCAAT sequence (see fig 1). This fact offered us the opportunity to test the relevance of this box in determining a cold shock enhancement of *hns* expression. Thus, we fused the promoter of the *Proteus* gene to a promoter-less *cat* gene and investigated the cold inducibility of the expression of this construct in comparison with that displayed by an equivalent construct made with *E coli hns* promoter. As seen in figure 9, when grown at 37°C, *E coli* cells transformed with the *P vulgaris hns-cat* fusion expressed a detectable amount of CAT activity (time 0); this level, however, is more than one order of magnitude lower than that expressed by cells transformed with the *E coli hns* fusion indicating that the heterologous promoter is recognized by the transcriptional machinery of the host, albeit with a lower efficiency. The low activity of the *Proteus hns* promoter in *E coli* may explain our previous finding that it was possible to transform *E coli* cells with a high copy number vector carrying the entire *Proteus hns* gene, while a similar construct made with the homologous *E coli hns* gene was lethal [13, 17, 18]. After cold shock, the level of CAT activity expressed *in vivo* by cells transformed with either the *P vulgaris* or the *E coli hns-cat* fusion displayed a clear-cut increase starting approximately 2 h after the temperature shift. Compared to the increase of CAT expression seen with the *E coli* fusion, the cold shock enhancement displayed by the *Proteus* fusion was actually much higher; this is probably due to the above-mentioned higher basal (37°C) level of expression of the *E coli* construct. Thus, it can be concluded from this result that the presence of a

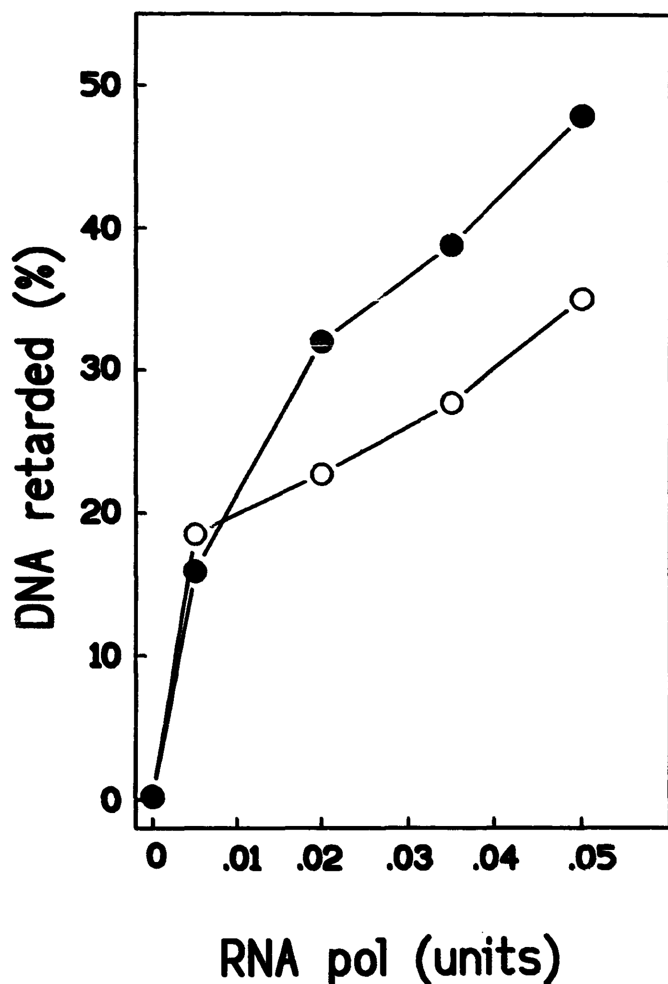

Fig 8. Electrophoretic mobility shift of the 110-bp promoter region by increasing amounts of RNA polymerase in the presence (•) and absence (○) of CS7.4 (3.9 µM). The experimental conditions are essentially similar to those described in figure 3 but for the fact that the samples contained 10 mM $MgCl_2$ and 50 mM KCl and the amounts of RNA polymerase indicated in the abscissa. Furthermore the samples were subjected to electrophoresis in Tris-acetate buffer (40 mM Tris-acetate, (pH 7.4), 1 mM EDTA, 5 mM Na acetate) and the extent of gel shift quantified using a BioRad GS-250 Molecular Imager.

conserved CCAAT box is not strictly required for the cold shock enhancement.

Discussion

The major *Escherichia coli* cold shock protein CS7.4 (CspA), homologous to the class of eukaryotic DNA-binding proteins recognizing the CCAAT-box [2, 8] was found to act as transcriptional activator of at least two genes, *hns* [6] and *gyrA* [7], belonging to the cold shock stimulon. In the present paper, we have investigated further the interaction of CS7.4 with the *hns* promoter and have shown that all or nearly all the elements necessary for the interaction of CS7.4 with *hns* and for its stimulation of transcription lie within the proximal 110 bp DNA fragment of the gene. In fact: a) an extract of cold shocked cells caused gel shifts of both a 660 bp and a 110 bp DNA fragment containing the *hns* promoter with similar efficiency; b) the same DNA fragments of *hns* bound to solid support were found to retain the CS7.4 protein with similar affinity; c) transcription-translation systems prepared with cell-free extracts of cold-shocked cells were found to produce the same stimulation of CAT synthesis when programmed with a promoter-less *cat* gene fused to either the 110 bp or the 660 bp fragment of *hns*.

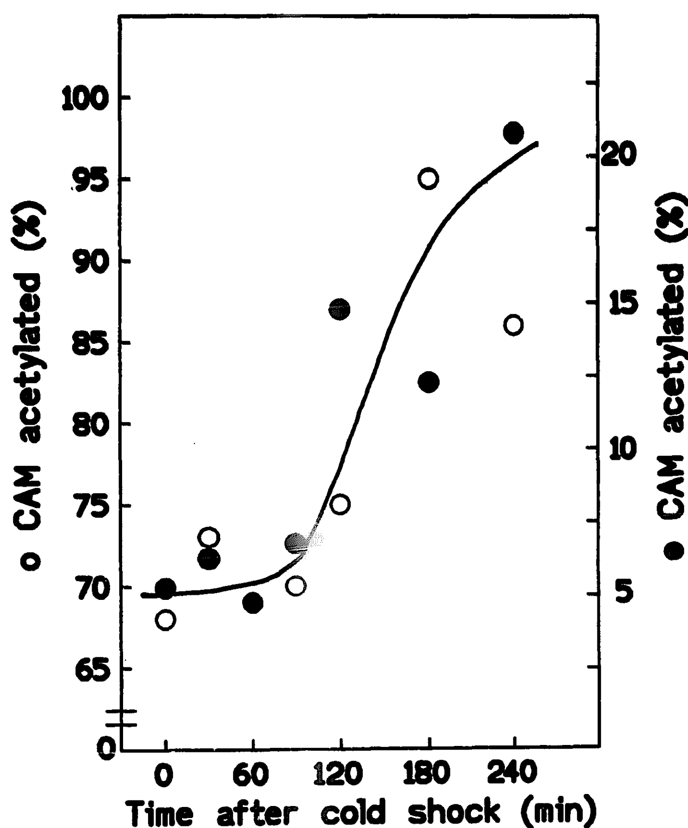

Fig 9. Cold shock induction of CAT activity in *E coli* cells transformed with *hns-cat* fusions containing either *P vulgaris* or *E coli hns* promoter. *E coli* DH5 cells transformed either with pKK110 (o) containing the *E coli hns-cat* fusion (○) [6] or with pKK250prot containing the *P vulgaris hns-cat* fusion (•) were grown in LB at 37 °C to A_{620} = 0.8. Samples were withdrawn at the indicated times after the shift to 10°C to prepare cell extracts to be tested for CAT activity as previously described [6].

Our experiments have also shown that, in contrast to the specific and efficient interaction of CS7.4 with its DNA target seen in the presence of crude cell extracts, purified CS7.4 bound to DNA with low affinity, at least judging from gel shift and DNase I footprinting experiments. This was seen with a large number of independent preparations of CS7.4 and may be due, at least in part, to instability of the purified protein but may also be due to the fact that some additional factor is required for efficient binding of CS7.4 to DNA. This factor cannot be one of the major DNA-binding proteins (*eg* H-NS and HU), since these had been removed from the cell extracts by passage through DNA cellulose. Our results suggest, on the other hand, that an important role may be played by the RNA polymerase. In fact, the footprinting experiments (figs 6, 7) indicated that most of the effects of CS7.4 (increased and decreased accessibility to DNase I at different sites) are seen in the presence of RNA polymerase and, in turn, the gel shift experiment showed that CS7.4 stimulated the binding of RNA polymerase to the promoter region (110 bp fragment) of *hns* (fig 8). It should be recalled that the 3D structure of CspB, the *Bacillus subtilis* homologue of CS7.4 showed significant similarities to SSB protein [4] while gel shift experiments demonstrated the preferential binding of this protein to single-stranded DNA compared to double-stranded DNA [5]. Taken together, our results and these observations suggest that the formation of the open complex by the RNA polymerase may favor the binding of CS7.4 to the promoter region of *hns* with the cold shock protein interacting preferentially with its target in a single-stranded conformation and, in turn, stimulating the activity of the RNA polymerase by favoring the melting of the duplex. Compatible with this interpretation is the fact that the main effect of CS7.4 seen in the footprinting experiment is the induction of an increased accessibility at several sites at and near the −10 and −35 elements of the promoter as well as downstream from the transcriptional initiation site, notably in the region between +4 and +20 which contains the cold shock box CCAAT overlapping a CG clamp (the CCACCCC sequence from +7 to +13). This structure may hinder the opening of the duplex, especially at low temperature, and may therefore account for the stimulation of *hns* transcription by CS7.4. While the relevance of this sequence is presently under study, in this work we have investigated the stringency of the CCAAT motif in determining the cold shock induction of *hns*. It should be recalled, in this connection, that even though the three CCAAT boxes present in *gyrA* were found to be necessary for the binding of CS7.4 protein to DNA [7], a CCAAT element has not been found in the promoter of all cold shock genes and that this sequence is not fully conserved in other cases (*eg* in *nusA* there is a CCAAA sequence between the −10 and −35). Since the *hns* promoter region of *P vulgaris* is unique among the Enterobacteriaceae *hns* sequences known sofar in that it does not contain a conserved CCAAT sequence [13], we investigated the cold shock inducibility of *cat* expression when placed under the control of this promoter and found that the CAT activity expressed from this fusion increases over fourfold after 4 h of cold shock. Thus, we can conclude that a conserved CCAAT element is not stringently required for cold shock activation. It remains to be established whether these results imply that the CCAAT sequence is dispensable for the binding of and transcriptional stimulation by the CS7.4 protein or whether they mean that there is more than one mechanism of cold shock stimulation of gene expression.

Acknowledgments

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