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The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor

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The H-NS protein is a major constituent of the *Escherichia coli* nucleoid structure and is implicated in the compact organization of the chromosome. Based on recent genetic evidence, this protein appears to influence the transcription of a variety of apparently unlinked genes on the chromosome, although the underlying molecular mechanism is not fully understood. In this study, we carried out a series of *in vitro* transcription assays including purified H-NS with special reference to the osmotically inducible *proV* promoter of the *proVWX* operon (or *proU*), whose expression is known to be derepressed by lesions of the *hns* (*osmZ*) gene. Here, H-NS was revealed to selectively inhibit an early step(s) of *proV* transcription initiation through its direct binding to the promoter region. It was thus demonstrated that H-NS functions directly as a transcriptional repressor. Under the *in vitro* conditions used, this *in vitro* inhibitory effect of H-NS was affected by changes in the superhelical density of template DNAs and more significantly by the concentration of potassium (K^+) ions. These results are also discussed with regard to the mechanism underlying regulation of the *proV* promoter in response to the medium osmolarity.

Key words: DNA curvature/DNA supercoiling/*E.coli* nucleoid protein H-NS/osmotic gene regulation/transcriptional repressor

Introduction

Although the chromosomal structures of eukaryotic and prokaryotic cells seem to be very different, a subset of *Escherichia coli* DNA-binding proteins have been implicated in the compact organization of chromosomal DNA, i.e. the nucleoid structure (Drlica and Rouviere-Yaniv, 1987; Pettijohn, 1988; Schmid, 1990). Among these proteins, the most abundant and best characterized are HU and H-NS (or H1a), whose functions have been the subject of longstanding debate. H-NS was first identified many years ago (Varshavsky *et al.*, 1977; Spassky *et al.*, 1984) and was recently confirmed to be a major component of the nucleoid by means of immunoelectron microscopy (Durrenberger *et al.*, 1991). This protein is a neutral, homodimeric protein comprising 136 amino acids. Its coding-gene, *hns*, was recently cloned and mapped at 27 min on the *E.coli* genetic map (Pon *et al.*, 1988; Göransson *et al.*, 1990; Hulton *et al.*, 1990; May *et al.*, 1990). Although this protein has been characterized as a relatively non-specific DNA-binding protein, one of the notable features of H-NS is that it induces strong condensation of DNA, without significantly affecting its topological properties (Spassky *et al.*, 1984). In spite of these and other intensive biochemical studies, however, it has been difficult to ascribe a physiological function to H-NS in the absence of genetic studies.

Recent genetic data pointed to the importance of H-NS in transcriptional regulation. Regulatory mutations in a number of different genes (*drdX*, *bglY*, *osmZ*, *pilG* and *virR*) appear to be allelic to *hns* (Göransson *et al.*, 1990; Higgins *et al.*, 1990, and references therein; Hulton *et al.*, 1990). Thus, H-NS appears to influence the regulation of a subset of apparently unlinked genes. Consistent with this view, the expression of a large number of *E.coli* cellular proteins is affected in a *hns* deletion background, as compared with the levels in wild-type cells (Bertin *et al.*, 1990; Yamada *et al.*, 1991). In most cases, mutational lesions of H-NS cause an increase in transcription (i.e. derepression), so H-NS presumably acts as a negative regulator of transcription. Based on these genetic data, two models have been proposed, in which the mechanism underlying global gene regulation by H-NS is explained as either 'changes in DNA supercoiling of chromosomal DNA' or 'transcriptional silencing of chromosomal DNA' (Göransson *et al.*, 1990; Higgins *et al.*, 1990). In either case, nothing is known about the underlying molecular mechanism. Therefore to address this particular issue as to the possible functions of H-NS, we carried out a series of *in vitro* transcription assays with purified H-NS. For this purpose, we chose one of the best characterized *E.coli* genes, namely the *proVWX* operon (practically called *proU*), whose expression is clearly derepressed by certain lesions of *hns* (*osmZ*) (Higgins *et al.*, 1988; Hulton *et al.*, 1990).

The *proVWX* operon encodes a high affinity transport system for glycine-betaine (Csonka, 1982; May *et al.*, 1986; Gowrishankar, 1989). Expression of this operon is remarkably stimulated in response to hyperosmotic stress (Cairney *et al.*, 1985; Dunlap and Csonka, 1985; Gowrishankar, 1985; Barron *et al.*, 1986; May *et al.*, 1986). The putative promoter of this operon, including its *cis*-acting regulatory sequences, has been extensively characterized by several groups (Gowrishankar, 1989; May *et al.*, 1989; Overdier *et al.*, 1989; Park *et al.*, 1989; Stirling *et al.*, 1989; Dattananda *et al.*, 1991; Lucht and Bremer, 1991; Overdier and Csonka, 1992). Since the results of these studies indicated that a major promoter is located upstream of the first gene of this operon, *proV*, the term '*proV* promoter' is used hereafter in this text. Here, it was demonstrated that H-NS functions as a selective transcriptional repressor for *proV* expression through its direct binding to the promoter region. Under the *in vitro* conditions used, such an inhibitory effect of H-NS was found to be affected by the concentration of K^+ and the superhelical density of template DNAs. These results are also discussed in relation to the mechanism underlying

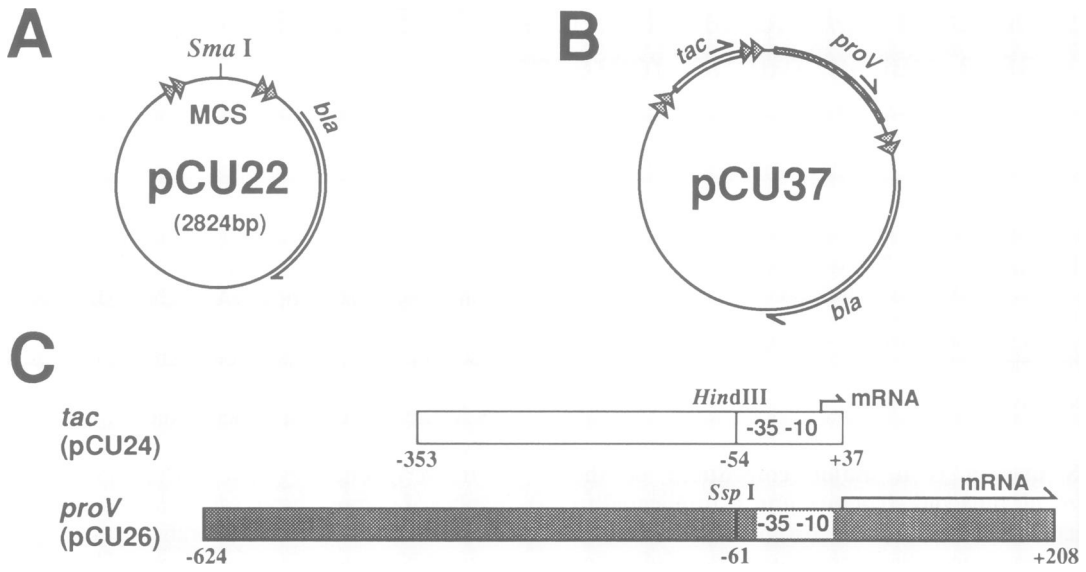

Fig. 1. Schematic structures of plasmids used for *in vitro* transcription assay. **A**. Structure of the plasmid vector, pCU22, used for cloning of promoter fragments. The shaded triangles represent *rho*-independent transcription termination signals, which are expected to terminate transcriptions proceeding to the direction of triangles. This plasmid contains the multi-cloning sites (MCS) that were derived from pUC19. **B**. Schematic structure of the plasmid, pCU37, which carries both the *tac* and *proV* promoter fragments, is shown. Detailed structures of these promoter fragments are given in panel C. **C**. Structures of the promoter fragments used in this study are schematically shown. The names of plasmids carrying the respective promoter fragments are indicated in the parentheses. Nucleotide numbers are also indicated (the transcription initiation sites was taken as +1).

regulation of the *proVWX* operon in response to hyperosmotic stress.

Results

Experimental design for in vitro transcription

Based on previous genetic evidence, one can envisage at least two alternative models with regard to the possible function of H-NS; namely it may somehow indirectly affect *proV* expression through unknown mechanisms or alternatively, it may function as a transcriptional repressor through its direct binding to the *proV* promoter. To examine the latter possibility in a rather direct way, we carried out a series of *in vitro* transcription assays with purified *E.coli* RNA polymerase as well as H-NS. Considering the fact that the DNA molecule in *E.coli* cells is negatively supercoiled, plasmid DNAs with a naturally occurring superhelical density were used as templates for the *in vitro* transcription assay. First, a versatile plasmid vector was constructed, as shown in Figure 1A (pCU22). This particular plasmid contains multi-cloning sites (MCS), which are flanked at both ends by *rho*-independent transcription termination signals, each of which is composed of tandemly arranged termination signals (for details, see Materials and methods). Any DNA segment cloned into the multi-cloning sites of this plasmid can be examined as to its *in vitro* transcription ability, since this plasmid template would allow us to detect RNA transcripts of relatively short and discrete lengths in nucleotides (see Figure 2). Taking advantage of this plasmid, a set of promoter-probing plasmids was constructed, particularly for the *proV* and *tac* promoters, as shown in Figure 1C. Plasmid pCU26 thus contains a segment encompassing the *proV* promoter in its proper orientation with respect to the termination signals, whereas pCU24 contains the *tac* promoter (Ueguchi *et al.*, 1992). The *proV* promoter sequence characterized here extends from nucleotide position −624 to +208 (see Figure 1C, the transcription starting site was taken as +1), which contains most, if not all, of the *cis*-acting regulatory regions required for *proV* expression (Gowrishankar, 1989; May *et al.*, 1989; Stirling *et al.*, 1989). The latter promoter, *tac*, was used throughout this study as a negative reference, since the expression of this promoter appears to be *hns*-independent.

H-NS functions as a transcriptional repressor for the proV promoter

Supercoiled template DNAs were purified from *E.coli* cells transformed with the respective plasmids, pCU24 and pCU26. An equal amount of each template DNA was mixed together and preincubated with H-NS and then subjected to an *in vitro* single round transcription assay (Figure 2). Among several transcripts of different nucleotide lengths, we could easily identify each transcript, namely, *proV*, *tac* and RNA-I, on the basis of the sizes predicted from their nucleotide sequences. The latter transcript, originated intrinsically from the vector DNA used (Tomizawa *et al.*, 1981). It should be noted that two major species of RNA transcripts were detected for each promoter, *proV* and *tac* (the longer ones are indicated by triangles in Figure 2). This appears to be due to that transcription from the respective promoters terminated at tandemly arranged termination signals separated by ~40 bp. However, as their transcription generally terminated at the upstream signal, we paid more attention to the major (shorter) one for quantitative analyses. In any case, it was revealed that upon preincubation of the supercoiled template DNA with increasing amounts of H-NS, the *proV* transcription was progressively inhibited, while the *tac* transcription was not inhibited at all (lanes 1−7). It is also worth mentioning that the level of RNA-I transcription was also unchanged. This transcript appears to be another fortuitous and negative reference. It was thus suggested that H-NS exhibits an inhibitory effect specific for *proV* transcription under the *in vitro* conditions used.

It has been reported that the binding of H-NS to DNA

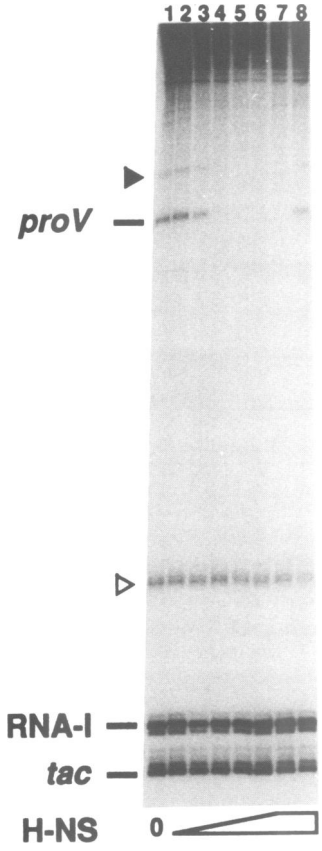

Fig. 2. Autoradiogram showing *in vitro* transcription assay in the presence of H-NS. Supercoiled plasmid DNAs (pCU24 and pCU26, 0.15 pmol each) were mixed and preincubated with varied amounts of H-NS and then subjected to *in vitro* single round transcription assay, under the conditions given in Materials and methods. Radioactive transcripts were separated on 8 M urea−6% acrylamide gel electrophoresis, followed by autoradiography. Each transcript was identified as indicated. The shaded and open triangles indicate the minor transcripts, which were originated from the *proV* and *tac* promoters, respectively. The amounts of H-NS used in lanes 1−7 were 0, 1.2, 6, 30, 40, 50, 60 pmol, respectively. Note that in lane 8, 60 pmol of H-NS was added after the incubation of the template DNAs with RNA polymerase.

molecules results in compaction of the DNA (Spassky *et al.*, 1984). We thus suspected that the observed inhibitory effect of H-NS may be due to such non-specific compaction of the whole template DNA used, which would somehow render the template DNA transcriptionally inactive. To address this issue, we constructed another plasmid, pCU37, in which both the *proV* and *tac* promoters were placed next to each other, i.e. the two promoters are located closely on the same template molecule (see Figure 1B). As shown in Figure 3, even when pCU37 was used as a template, essentially the same *proV*-specific inhibitory effect was observed, while *tac* transcription originating from the same template was never affected (lanes 1−3). This result suggested that the inhibitory effect of H-NS on the *proV* transcription is a promoter-specific and highly local event and not due to non-specific inactivation of the whole template DNA.

It was further revealed that when the template DNA was first incubated with RNA polymerase, followed by incubation with H-NS, the inhibitory effect on the *proV* transcription was less evident (Figure 2, lane 8). It should be noted that we repeated essentially the same experiment several times and confirmed that when H-NS was added after

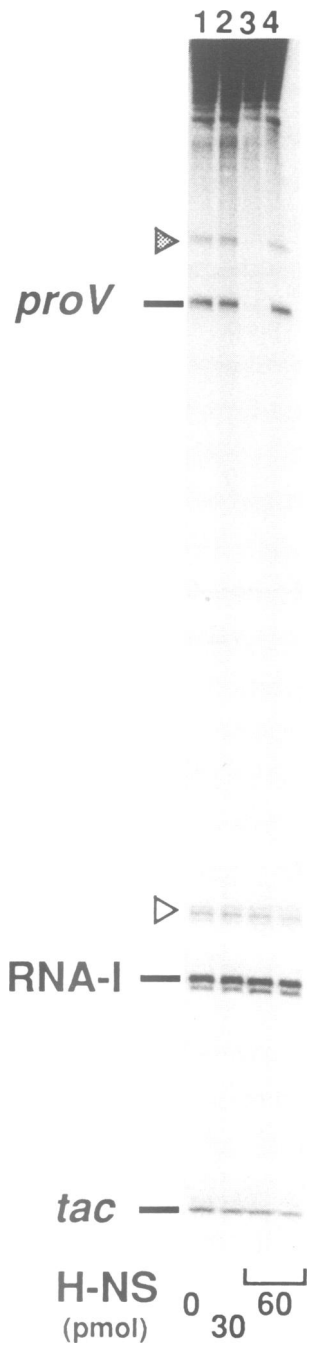

Fig. 3. Autoradiogram showing *in vitro* transcription assay with use of pCU37 as a template. Supercoiled DNA (pCU37, 0.3 pmol) was preincubated with the indicated amounts of H-NS and then subjected to *in vitro* transcription assay under the same conditons given in Figure 2. Note that in lane 4 the template DNA was first incubated with RNA polymerase for 1 h and then with H-NS. Note also that the amount of the template DNA used in this experiment was 2-fold higher than that used in Figure 2.

preincubation of template DNAs and RNA polymerase, no significant inhibitory effect on the *proV* transcription was observed (if any, it was within the experimental error) (see also Figure 3, lane 4). These results suggested that H-NS affects an early step(s) of transcription initiation such as 'closed or open complex formation', rather than subsequent steps such as 'RNA chain elongation'. Taking all these results together, it was concluded that H-NS appears to function

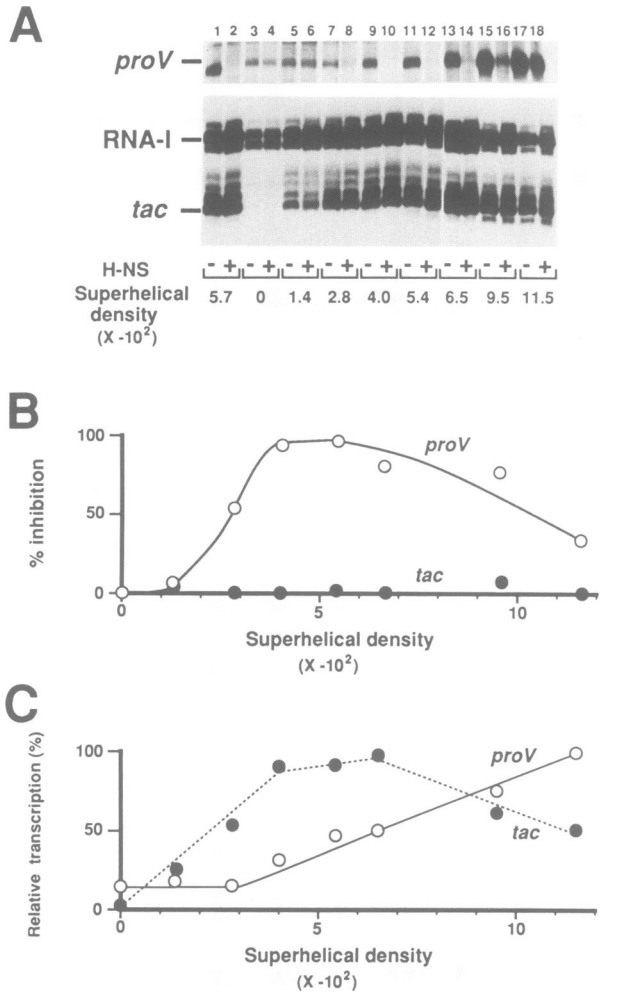

Fig. 4. Autoradiogram showing *in vitro* transcription assay with use of template DNAs with varied superhelical densities. **A.** *In vitro* transcription assay was carried out by using the mixed template DNAs (pCU24 and pCU26, 0.15 pmol each), in which each template DNA had a different superhelical density; naturally occurring (lanes 1 and 2) and artificially changed (lanes 3−18) superhelical densities. These template DNAs were preincubated either in the absence (odd lanes) or presence (even lanes) of 60 pmol of H-NS. Other details are the same as those given in Figure 2. **B.** The relative amounts of each transcript were measured on the basis of the autoradiogram shown in panel A. The degree of inhibition by H-NS was calculated both for the *proV* and *tac* transcripts, and shown as the function of superhelical density. **C.** The relative amounts of the *proV* and *tac* transcripts in the absence of H-NS were also measured, and shown as the function of superhelical density. The maximum amounts determined for the *proV* (lane 17) and *tac* (lane 13) were taken as 100%, respectively.

as a selective transcriptional repressor, in this case, for the *proV* promoter.

Effect of the superhelical density on the inhibitory effect of H-NS

In the experiments described above, we used template DNAs with a naturally occurring superhelical density, which were purified from cells grown in certain medium, particularly Luria-broth. However, considering the previous notion that *proV* expression *in vivo* is influenced by changes in the level of supercoiling of the chromosomal DNA (Higgins *et al.*, 1988), we next examined whether or not the *in vitro* inhibitory effect of H-NS is affected by the superhelical density of the template DNA used. To address this issue,

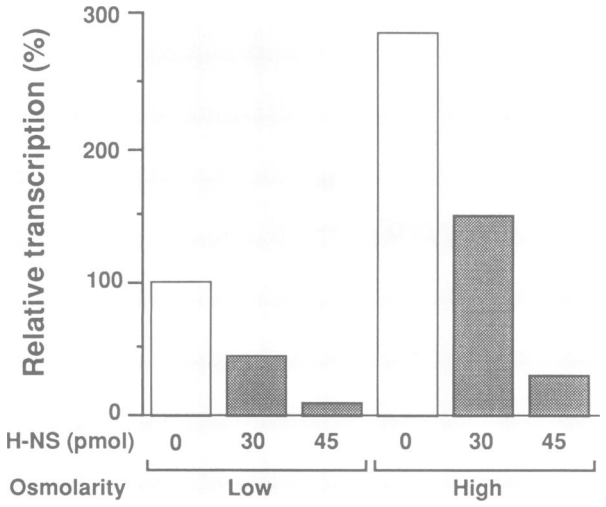

Fig. 5. Inhibitory effect of H-NS on the *proV* transcription with template DNAs isolated from the media of low and high osmolarity. Plasmids pCU24 and pCU26 were isolated from cells grown in Luria-broth, in which NaCl was omitted (low osmolarity) or supplemented to the final concentration of 0.5 M (high osmolarity). After preincubated of these template DNAs in the absence (open bar) or presence (shaded bar) of H-NS (30 or 45 pmol), *in vitro* transcription assay was carried out under the same conditions given in Figure 2. The relative amounts of the *proV* transcript were measured (the value determined for the one from the low osmolarity medium in the absence of H-NS was taken as 100%).

the same purified plasmid DNAs as used in Figure 2 were treated with a eukaryotic topoisomerase in the presence of various concentrations of ethidium bromide to prepare a set of template DNAs with various superhelical densities (Singleton and Wells, 1982). The superhelical density of each template thus obtained was first measured (see Materials and methods). Then, using these template DNAs, an *in vitro* transcription assay was carried out (Figure 4A). The results are presented in a quantitative manner as a function of the superhelical density determined for each template (Figure 4B). It was revealed that the *proV*-specific inhibitory effect of H-NS was somewhat dependent on the superhelical density of the template used (for example, compare lanes 5 with 6, and 11 with 12). In other words, the inhibitory effect was less evident for the template DNAs with superhelical densities < -0.028 or > -0.095 (Figure 4A and B). It should be noted again that neither the *tac* nor RNA-I transcription was ever affected by H-NS at any superhelical density tested. Interestingly, it was also found that the overall level of *proV* transcription itself in the absence of H-NS was dependent on the superhelical density (Figure 4C). This was also observed for the *tac* transcription.

Inhibitory effect of H-NS and osmotic regulation

We then addressed the issue: whether or not the superhelical density-dependent inhibitory effect of H-NS, observed *in vitro*, is physiologically relevant. Plasmid DNAs were isolated from cells grown in media of low and high osmolarity. Their superhelical densities were determined to be -0.033 (low osmolarity) and -0.054 (high osmolarity), respectively. These results were consistent with the previous notion that the naturally occurring superhelical density of DNA isolated from *E.coli* cells somehow varies in response to changes in the medium osmolarity (Higgins *et al.*, 1988). Using these template DNAs, we carried out an *in vitro* transcription assay, the results being presented in a

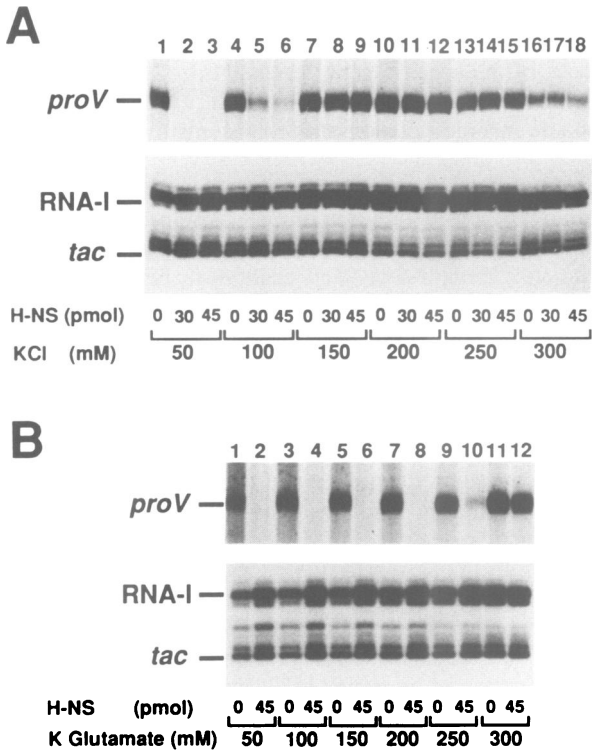

Fig. 6. Effect of potassium salts on the inhibitory effect of H-NS. Supercoiled template DNAs (pCU24 and pCU26, 0.15 pmol each) were mixed and preincubated with the indicated amounts of H-NS in the presence of the indicated concentrations of potassium salts and then *in vitro* single round transcription assay was carried out under the same conditions given in Figure 2. In panels A and B, potassium chloride and potassium glutamate were used, respectively.

quantitative manner in Figure 5. It was revealed that the inhibitory effect of H-NS was observed for both the templates and nearly to the same extent. It was thus considered that although it is clear that H-NS functions as a repressor for the *proV* promoter in a superhelical density-dependent manner, the naturally occurring variation in the superhelical density in cells in response to the medium osmolarity is not striking enough to support fully the idea that physiological changes in the superhelical density affect the inhibitory effect of H-NS.

Effect of potassium ions on the inhibitory effect of H-NS

Another parameter proposed previously as a signal for *proV* expression is the intracellular concentration of K^+ (Sutherland *et al.*, 1986; Jovanovich *et al.*, 1989; Ramirez *et al.*, 1989; Prince and Villarejo, 1990). Furthermore, it is known that the intracellular concentration of K^+ is known to increase from ~100 to 400 mM in response to an osmotic upshift of the growth medium (Epstein and Schultz, 1965; Laimins *et al.*, 1981; Sutherland *et al.*, 1986). It should, however, be noted that we used 100 mM KCl for the present series of *in vitro* transcription assays. To gain an insight into the possible role of K^+ in the *proV* transcription as well as in the inhibitory effect of H-NS, we carried out an *in vitro* transcription assay in which various concentrations of KCl were supplied (Figure 6A). The *proV* transcription per se was slightly affected when the concentration of KCl was varied (compare lanes 1, 4, 7, 10, 13 and 16). Quantitative analysis revealed that the level of *proV* transcription at 200 mM KCl (lane 10) was ~1.7-fold higher than that at 50 mM (lane 1). In any event, the *proV*-specific repression by H-NS was abolished when a high concentration of KCl (>150 mM) was added. To confirm further this particular effect of K^+ ions, potassium glutamate (instead of KCl) was used for the *in vitro* transcription assay (Figure 6B). The *proV* transcription was more significantly affected when the concentration of potassium glutamate was varied, i.e. the *proV* transcription observed at 300 mM potassium glutamate (lane 11) was ~4-fold higher than that at 50 mM (lane 1). Importantly, it was found that the inhibitory effect of H-NS in either case (KCl or potassium glutamate) was strikingly sensitive to the concentration of K^+, i.e. the *proV*-specific repression by H-NS was abolished when a high concentration of K^+ was added, although the effective concentration differed between the cases of KCl and potassium glutamate. These results imply two possible effects of K^+ on the *proV* transcription. One is activation of the *proV* transcription per se and the other is the modulation of the inhibitory effect of H-NS.

Even when potassium glutamate was used for the *in vitro* transcription assay, H-NS exhibited no inhibitory effect on both the *tac* and RNA-I transcription at any concentration of K^+ used (Figure 6B). It would also be worth mentioning that under these particular conditions, the promoter activities observed for *tac* and RNA-I appear to be weaker than that for *proV* (as judged from the intensity of each band on the autoradiogram and the number of radioactive U-residues incorporated into each transcript). These observations argue against the possibility that H-NS non-specifically inhibits transcription only at weak promoters while transcription at strong promoters is insensitive to H-NS. Thus, they further confirmed that *proV* transcription is selectively inhibited by H-NS.

Discussion

The physiological function of the nucleoid protein, H-NS, has been the subject of longstanding debate (see Introduction). In this study, we demonstrated *in vitro* that H-NS directly and selectively inhibits transcription at the *proV* promoter. The results presented in this study strongly support the view that H-NS can function as a transcriptional repressor through its direct binding to the target promoters and inhibits an early step(s) of transcription initiation, namely closed and/or open complex formation. The latter view is consistent with the previous notion as to the *in vitro* effect of H1a (or H-NS) on the *lac* promoter function (Spassky *et al.*, 1984). It should, however, be emphasized that its mode of action appears to be different from those of many other sequence-specific transcriptional repressors in several aspects, as will be discussed.

Based on the finding that mutations in the *hns* (*osmZ*) gene result in not only derepression of the *proV* gene but also change in the linking number of reporter-plasmids, H-NS was considered to affect the expression of a subset of genes in an indirect manner via alteration of the supercoiling state of DNA (Higgins *et al.*, 1990; Hulton *et al.*, 1990). However, this attractive hypothesis is not supported by our data. First, H-NS can specifically inhibit transcription from the *proV* promoter when added to a simple *in vitro* system in which the only protein component is RNA polymerase holoenzyme and the template used is highly purified

supercoiled DNA, i.e. in the absence of topoisomerases and other nucleoid proteins such as HU. Secondly, it is known that H-NS itself exhibits little *in vitro* effect on the linking number of covalently closed DNAs (Spassky *et al.*, 1984). Our data rather suggest that the inhibition of *proV* transcription by H-NS is most likely a direct consequence of binding of H-NS to a target sequence(s) in the promoter region. Another previous proposal concerning the H-NS function, namely the 'transcriptional silencing model', is that H-NS may condense chromosomal DNA into a eukaryotic chromatin-like structure, rendering parts of the chromosome transcriptionally inactive (Göransson *et al.*, 1990). In this study, however, it was demonstrated that H-NS can selectively inhibit *proV* transcription, even when the *proV* and *tac* promoters are closely placed on a single template molecule. It is thus probable that the inhibitory effect of H-NS can take place within a relatively local region of the template DNA, suggesting that H-NS does not necessarily render a large portion of DNA transcriptionally inactive.

Although the molecular mechanism underlying the inhibition of *proV* transcription by H-NS is not clear at present, it would be of interest to address the issue of whether or not H-NS could be envisaged as an ordinary sequence-specific repressor. Apparently, this view contradicts the fact that H-NS was characterized as a relatively non-specific DNA-binding protein. In this respect, we recently demonstrated that H-NS exhibits relatively high affinity to the DNA segment encompassing the upstream sequence of the *proV* promoter (nucleotides extending −360 to −83) (Tanaka *et al.*, 1991a). Our preliminary DNase I footprinting analysis with this DNA segment revealed that H-NS gave a complex protection profile covering >150 bp region extending upstream from the −35 region (K.Tanaka, C.Ueguchi and T.Mizuno, unpublished data). Based on the *in vivo* analyses of the *proV* promoter, it was also proposed by other investigators that a negative regulatory element for *proV* expression is located downstream of the *proV* promoter (nucleotides extending to +274) (Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992). It is therefore most likely that a relatively large portion surrounding the canonical *proV* promoter region is required as the putative target site(s) of H-NS. These observations do not support the idea that H-NS functions through its binding to a short and specific nucleotide sequence. An alternative model is that H-NS may form a multimeric nucleoprotein complex around the *proV* promoter region, which would affect *proV* transcription by hindering the functioning of RNA polymerase. Consistent with this is the finding by Rimsky and Spassky (1990) that many molecules of H-NS bind cooperatively to the *lac* promoter DNA based on a loose consensus sequence. The crucial question then arose as to what is the specific determinant (or target) of H-NS binding. In this regard, it would be worth mentioning that H-NS preferentially recognizes a curved DNA sequence with relatively strong affinity *in vitro* (Bracco *et al.*, 1989; Yamada *et al.*, 1990; Owen-Hughes *et al.*, 1992). Indeed, it has been observed that upstream and downstream sequences of the *proV* promoter display a sequence-directed DNA curvature (Tanaka *et al.*, 1991a; Owen-Hughes *et al.*, 1992). It has been also suggested recently that a curved DNA element located downstream of the *proV* promoter is required for normal regulation of *proV* expression *in vivo* (Owen-Hughes *et al.*, 1992). It is thus tempting to speculate that such a DNA curvature may be a determinant involved in the inhibitory effect of H-NS. In any event, clarification of the molecular basis of the specific gene repression by H-NS must await further extensive genetical and biochemical studies, which should include isolation of H-NS-insensitive mutants of the *proV* promoter.

Several groups have addressed the mechanistic issue as to how *in vivo* expression of *proV* is remarkably enhanced in response to hyperosmotic stress. It has been proposed that osmotic strength-dependent changes in DNA supercoiling comprise the mechanism that controls *proV* expression (Higgins *et al.*, 1988). Then, other groups carried out several series of *in vitro* transcription studies on *proV* expression with special reference to its osmotic induction (Jovanovich *et al.*, 1989; Ramirez *et al.*, 1989; Prince and Villarejo, 1990; Ramirez and Villarejo, 1991). They argued against the supercoiling hypothesis and alternatively proposed that the concentration of intracellular K^+ plays a crucial role in the osmotic induction of *proV* (Ramirez and Villarejo, 1991). The results of our *in vitro* transcription experiments, in which the concentration of K^+ and the superhelical density were changed, suggested that the osmotic induction of *proV* could be achieved through synergistic effects caused by the elevated concentration of intracellular K^+ and the increased negative superhelical density in response to hyperosmotic stress. In any event, *in vivo*, if H-NS can strictly repress *proV* expression as observed *in vitro*, this effect must be conditional, i.e. it must be relieved in response to hyperosmotic stress. In this respect, our results demonstrated that the *in vitro* inhibitory effect of H-NS is somewhat dependent on the superhelical density of templates. However, it was suggested that the naturally occurring variation of DNA supercoiling in response to hyperosmotic stress may not be striking enough to support the idea that physiological changes in DNA supercoiling is mainly responsible for modulation of the inhibitory effect of H-NS. We rather found that the concentration of K^+ affect more strikingly the inhibitory effect of H-NS, i.e. H-NS no longer inhibits *proV* transcription when the concentration of K^+ is relatively high under the *in vitro* conditions used. Although our results do not rule out other mechanistic possibilities, for example, a covalent modification of H-NS in response to hyperosmotic stress through an unknown mechanism, it is reasonable to assume that the *in vivo* function of H-NS can be modulated directly by changes in the intracellular ionic composition, particularly K^+. This makes good sense as the *in vivo* mechanism by which a 100-fold osmotic induction of *proV* is achieved, when considered the previous notion that an increase in medium osmolarity results in rapid accumulation of intracellular K^+ (Epstein and Shultz, 1965; Laimins *et al.*, 1981; Sutherland *et al.*, 1986). When the medium osmolarity is relatively low, under which conditions the intracellular concentration of K^+ is also relatively low, H-NS can thus severely repress *proV* expression. As the medium osmolarity is increased, the concentration of K^+ and perhaps the negative supercoiling of DNA increase, which in turn results in stimulation of *proV* transcription per se and simultaneously results in relief of the repression by H-NS. Verification of this model awaits further extensive studies.

In conclusion, one of the physiological functions of the nulceoid protein, H-NS, was fairly well clarified to be as a transcriptional repressor. Furthermore, its mode of action

appears to be different from those of many other sequence-specific transcriptional repressors in several respects. Thus, as postulated previously based on genetic data, H-NS appears to play not only a purely structural role in the organization of the chromosome, but also a rather dynamic role in the regulation of gene expression. Thus, H-NS may provide us with a unique paradigm of global transcriptional regulation in prokaryotes.

Materials and methods

Bacteria and media

A bacterial strain MV1184 (Vieira and Messing, 1987), a derivative of *E.coli* K-12, was mainly used as a host strain for manipulation of plasmid DNAs. Cells were cultivated in Luria-broth (Miller, 1972) containing 50 μg/ml of ampicillin, if necessary NaCl was omitted (for low osmolarity) or supplemented to the final concentration of 0.5 M (for high osmolarity).

Materials

H-NS protein was purified as described previously by Tanaka *et al.* (1991b). *E.coli* RNA polymerase was purchased from Boehringer Mannheim. [α-^{32}P]UTP (30 TBq/mmol) was obtained from Amersham International. Restriction endonucleases and modification enzymes were from Takara Shuzo Co. Ltd or Toyobo Co. Ltd. All other materials were of reagent grade.

Plasmid construction

Plasmid pCU22, which was used as a versatile vector for a series of *in vitro* transcription assay, was constructed as follows. Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was completely digested with *Hin*dIII and then partially with *Vsp*I, a 126 bp region encompassing the lactose promoter, which was to be removed. The linealized DNA was blunt-ended by T4 DNA polymerase and then self-ligated. Note that the unique *Hin*dIII site was recreated in the resultant plasmid, pCU12. A 128 bp *Bam*HI−*Bgl*II fragment containing *rho*-independent transcriptional termination signals was purified from plasmid pVBR-A (a gift from K.Shigesada, Kyoto University), followed by treatment with T4 DNA polymerase. Then, this fragment was inserted into the previously blunt-ended *Hin*dIII site of pCU12, plasmid pCU21 being yielded. The same fragment as described above was inserted into the previously blunt-ended *Eco*RI site of pCU21 in order to construct pCU22, in which two sets of trasncriptional termination signals were placed (see Figure 1A). Plasmid pCU24 was constructed previously (Ueguchi *et al.*, 1992, and see Figure 1C). To clone the *proV* promoter into pCU22, a 855 bp *Kpn*I−*Pst*I fragment encompassing the *proV* promoter sequence (−624 to +208), which was flanked by a short multirestriction sequence (23 bp) from pUC19, was isolated from p445-05 (K.Tanaka, C.Ueguchi and T.Mizuno, unpublished). After treatment of this fragment with T4 DNA polymerase, it was inserted into the unique *Sma*I site of pCU21 and pCU22, the resultant plasmids being designated as pCU25 and pCU26, respectively. Plasmid pCU37 was constructed as follows: a blunt-ended 1017 bp *Xho*I−*Kpn*I fragment encompassing the *proV* promoter and its upstream termination signals was isolated from pCU25, and then ligated with the previously blunt-ended *Kpn*I site of pCU24 (see Figure 1B).

In vitro transcription assay

The conditions used for *in vitro* transcription assay were essentially the same as those described previously by Ueshima *et al.* (1989). Supercoiled DNA was isolated by alkali-lysis procedure, and then purified by means of centrifugation in CsCl gradient containing ethidium bromide (Sambrook *et al.*, 1989). Note that the centrifugation was repeated twice in order to obtain highly purifed palsmid DNAs. Template DNAs thus isolated (0.15 pmol each) were mixed and incubated in the presence of appropriate amounts of H-NS at 37°C for 20 min in 32 μl of reaction mixture containing 50 mM Tris−HCl (pH7.8), 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25 μg/ml of bovine serum albumin, 50 mM NaCl and 100 mM KCl. If necessary, KCl concentration was varied or KCl was replaced by potassium glutamate. After addition of RNA polymerase (3 pmol), the samples were further incubated to form transcriptional open complex for 1 h. Then, single round RNA synthesis was started by adding 15 μl of substrates (NTPs)-heparin mixture containing 2 μCi of [α-^{32}P]UTP, followed by further incubation at the same temperature for 3 min. The reaction was stopped by the addition of a solution (50 μl) containing 40 mM EDTA and 300 μg/ml of yeast tRNA. The resultant transcripts were purified by phenol−chloroform extraction, and then precipitated in ethanol (70%, vol/vol). Samples were analyzed by 8 M urea−6% polyacrylamide gel electrophoresis, followed by autoradiography. Quantitative analyses of the autoradiograms were carried out with the aid of double beam densitometer (Shimadzu Co. Ltd, CS-9000)

Construction of a set of DNAs with varied superhelical densities

Covalently closed plasmid DNAs (5 μg) were treated with 6 units of calf thymus DNA topoisomerase I (Takara Shuzo Co. Ltd) in 100 μl of reaction mixture containing 35 mM Tris−HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine and 0.01% of bovine serum albumin. Note that such a reaction was carried out at 37°C for 4 h in the presence of varied concentrations of ethidium bromide (0−40 μM). DNAs were then purified by means of phenol−chloroform treatment twice, followed by ethanol precipitation. Mean linking difference of each DNA molecule ($\Delta\bar{L}k$) was measured by 0.7% agarose gels containing appropriate concentrations of chloroquine, according to the method of Keller (1975). The mean superhelical densities (σ) were calculated by the equation ($\sigma = 10\Delta\bar{L}k/N$, where N is the number of base pairs of DNA examined).

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