Interactions of the Nucleoid-associated DNA-binding Protein H-NS with the Regulatory Region of the Osmotically Controlled proU Operon of Escherichia coli*

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The Escherichia coli hns gene encodes the abundant nucleoid-associated DNA-binding protein H-NS. Mutations in hns alter the expression of many genes with unrelated functions and result in a derepression of the proU operon (proVWX) without abolishing the osmotic control of its transcription. We have investigated the interactions of H-NS with the proU regulatory region by deletion analysis of cis-acting sequences, competitive gel retardation assays, and DNase I footprinting. The negative effect of H-NS on proU transcription was mediated by cis-acting sequences within proV but did not depend on the presence of a curved DNA segment upstream of the proU -36 region previously characterized as a target for H-NS binding in vitro. We detected a 48-base pair high affinity H-NS binding region downstream of the proU promoter at the 5' end of the proV gene and a complex array of additional H-NS binding sites which suggest the presence of an extended H-NS nucleoprotein complex. Most of the H-NS binding sites were highly A+T-rich and carried stretches of 5 or more consecutive A-T base pairs. The implications of our results for the osmotic regulation of proU transcription are discussed.

Several DNA-binding proteins are assumed to play an important role for the organization of the Escherichia coli chromosomal DNA into a chromatin-like structure, the bacterial nucleoid (1–5). One of the most abundant nucleoid-associated proteins is H-NS (H1a–H1b), whose primary structure is highly conserved in Gram-negative bacteria (8–13). The H-NS protein from E. coli is a highly charged 137-amino acid residue polypeptide (M, = 15,500) that exists in solution predominantly as a homodimer due to strong hydrophobic interactions between the subunits (6). H-NS binds with high affinity but low sequence specificity to double-stranded linear or circular DNA and displays a certain preference for curved DNA segments (14–17). Binding of H-NS in vitro to circular closed plasmid DNA results in a compaction of its substrate without a strong effect on the linking number (5), and its overproduction in vivo results in a striking condensation of the bacterial chromosomal DNA (18). Several environmental factors and regulatory circuits control the intracellular concentration of H-NS. The amount of H-NS increases severalfold after a severe cold shock of the cells (19). Its structural gene, hns, is negatively auto-regulated, and hns transcription is enhanced when the culture enters stationary phase (5, 20, 21).

The cellular functions of H-NS have recently found widespread attention, since mutations in its structural gene show highly pleiotropic phenotypes. The hns gene has been genetically and physically mapped to the 27.5-min region of the E. coli chromosome (11–13, 22), and independently isolated mutations in this gene have been designated bglY, pilG, virR, and osm2 (for an overview, see Ref. 23). Mutations in hns lead to changes in gene expression (12, 15, 22, 24–30), influence genetic recombination (22, 31–33), affect bacterial virulence (9, 25), decrease the motility of the bacterial cell (34, 35), increase the formation of chromosomal deletions (36), and stimulate the transposition of bacteriophage Mu (37, 38). Certain hns alleles also cause alterations in the DNA supercoiling of reporter plasmids (17, 22, 25, 35). Both increases and decreases in the rate of synthesis of a sizable number of proteins are observed in hns mutants (34, 39). Several models have been suggested to explain the effects of H-NS, which is clearly not a classic sequence-specific regulatory protein, on gene expression. It might act indirectly, by influencing the supercoiling or the topological organization of chromosomal DNA, which could in turn lead to changes in transcription from DNA topology-sensitive promoters (17, 22, 23, 25, 35). Alternatively, H-NS might bind to DNA and affect gene expression directly, either as a transcriptional repressor for specific genes or as a "silencer" for extended chromosomal regions (13, 28, 38, 40–42). Experiments with the phage Mu repressor protein suggest that H-NS can also act through other, sequence-specific regulatory proteins by either facilitating their DNA binding or by stabilizing their protein-DNA interactions (37).

One of the best studied systems whose transcription is strongly affected by H-NS is the proU operon, which encodes a binding protein-dependent transport system for the osmoprotectant glycine betaine (43, 44). Transcription of the proU operon (proV, proW, and proX) is sensitively determined by the osmolarity of the environment. The basal transcription of proU is very low and is strongly stimulated upon a sudden osmotic upshock; its elevated level of transcription at high osmolarity is proportional to the osmolarity of the growth medium (45–49). One of the factors that determine the level of proU expression is the H-NS protein. Mutations in the hns structural gene result in a strong increase of proU transcription, but do not abolish osmoregulation of proU expression (12, 22, 25). Higgins and co-workers have suggested that proU transcription is regulated primarily through changes in the DNA supercoiling of the proU promoter region (11, 22, 23). In their model, H-NS would serve as a "scaffold" protein required for correct topological arrangement of the proU promoter sequences, hence it would act in
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DIRECTLY ON PROU TRANSCRIPTION (17). In contrast, Ueguchi and Mizuno (40) recently demonstrated that H-NS can selectively inhibit transcription of proU by E. coli RNA polymerase in vitro, which suggests that H-NS functions directly as a transcriptional repressor affecting early steps of transcription initiation.

In this report, we analyze the interaction of H-NS with DNA sequences both upstream and downstream of the proU promoter and examine the role of these sequences for the negative effect of H-NS on proU expression. By DNase I protection assays, we identify an extended H-NS binding region located at the beginning of the proV structural gene which is required for the normal, H-NS-dependent regulation of proU expression. Our data are in agreement with a direct repression model for proU transcription modulation by the H-NS protein.

EXPERIMENTAL PROCEDURES

Growth Conditions and β-Galactosidase Assays—Bacteria were grown aerobically at 37 °C in LBON medium. LBON is LB medium (50) prepared without NaCl (51). For β-galactosidase assays, cells were grown in LBON with different concentrations of NaCl. Specific β-galactosidase activity, expressed as micromoles of substrate (2-nitrophenyl β-D-galactopyranoside) cleaved/min/mg of protein, was determined as described elsewhere (41).

Bacterial Strains, Plasmids, and Phages—The bacterial strains described in this study are derivatives of the E. coli K-12 strain MC4100 (52). Strain MKH13 carries the pMP101/pMP2/pMP608 mutations which render it entirely deficient in glycine betaine uptake. Strain JM117 is a derivative of MKH13 and carries the hns-205-Tn10 insertion (21). The bla* (proV'-lacZ/xyb2) protein fusion plasmid pOS7 has been described (43, 53). Plasmids pA385, pA524, pA574, and pΔ574 are derivatives of pOS7 and carry progressive deletions of proU sequences upstream of the proV gene (26); their plasmid designation numbers correspond to the first proU base pair still present according to sequence designations upstream of the proV gene (26). Digestion of pOS7 with HindIII and Sau3A yields a 0.99-kb EcoRI-BstNI fragment from plasmid pA574 (Fig. 1) by digestion with EcoRI and BglI and labeled upstream of proU at the vector-derived EcoRI site; after the fill-in reaction it carries 7 bp of vector sequences upstream of proU.

Fragment II (−54 to +202 bp) was isolated from pΔ574-BalI (Fig. 1) by digestion with EcoO109I and BamHI and labeled downstream of the proU sequences at the BamHI site; after the labeling it carries 7 bp of vector sequences at the BamHI site and 24 bp at the EcoO109I site. Fragment III (−243 to +202 bp) was isolated from pΔ574 (Fig. 1) by digestion with EcoO109I and BglI and labeled upstream of the proU material at the EcoRI site; it carries 11 bp of vector sequences at the EcoRI end. DNA fragments (2.5 μg) were incubated with H-NS protein (0.075–6.2 μg) as described for the gel retardation assay. After protein-DNA complex formation, 25 ng of DNase I were added in 5 μl of a buffer containing 10 μM-Tris-HCl (pH 7.8), 12.5 mM CaCl2, 15 mM MgCl2, 1 mM dithiothreitol, and 200 mM NaCl. The digestion was stopped after 20 s by addition of 50 μl of 3.5 μM Na-EDTA (pH 8.0) containing 10 μg/ml yeast carrier RNA. The reaction products were then purified by phenol/chloroform extraction, precipitated with ethanol, and separated on a 6% sequencing gel. Sequencing reaction products (57) were produced with the Sequenase 2.0 kit and the −40 primer with phage M13mp18 DNA as the template (U. S. Biochemical Corp.); they were used as size standards on the gels with the reaction products of the DNase I protection assays. The proU sequence coordinates can be assigned for the DNase I reaction products since the DNA sequences of both the labeled proU DNA fragments used for the DNase I protection assays and that of phage M13mp18 are known (44, 53, 58).

RESULTS

H-NS BINDS TO THE PROU CONTROL REGION—DNA segments located upstream and downstream of the proU promoter are known to influence proU expression (17, 26, 51, 60). We performed competitive gel retardation assays with different DNA fragments derived from the proU regulatory region and purified H-NS protein. As substrate for the DNA binding assay we used restriction fragments of plasmid pOSt (Fig. 1). pOSt carries the proU promoter and a lacZ protein fusion to proV, the first gene of the proU operon, and is known to include all DNA sequences required in cis for complete osmoregulation of proU expression (26, 53). Digestion of pOSt with BglI yields six restriction fragments that compete for binding to H-NS and thus allowed us to monitor the relative affinity of the H-NS protein for each of the fragments (Fig. 1). It is evident that low protein concentrations, H-NS exhibited a preferential affinity for a 680-bp BglI fragment that carries the proU promoter and extends from position −475 to +202 bp relative to the transcription initiation site (Fig. 1). A small increase (approximately 2-fold) in H-NS concentration was sufficient to convert the majority of these DNA fragments from the free to the protein-bound form retarded in the gel, which suggests in agreement with previous data (61) a cooperative binding mode of H-NS. At
somewhat higher protein concentrations, H-NS also bound efficiently to a 1029- and a 577-bp BglI fragment (Fig. 1). These fragments carry part of the β-lactamase (bla) gene including its promoter and the fusion junction between the proV and lacZ genes present on plasmid pOSt, respectively (Fig. 1). A further increase of the H-NS concentration in the DNA binding assay first led to a “smearing” of all restriction fragments, and subsequently to the appearance of defined higher molecular weight DNA-protein complexes of each restriction fragment (Fig. 2A). Therefore, at high concentrations H-NS can bind to DNA fragments in a nonspecific fashion, but at low concentrations it shows a clear binding preference for the proU regulatory region.

A curved DNA segment is present upstream of the proU promoter (44), and a DNA fragment comprising this region (BENT-105; Fig. 1) has been shown to interact with purified H-NS in vitro (62). We asked whether the preferential interaction of H-NS with the 680-bp BglI restriction fragment from the proU regulatory region (Fig. 2A) was due to the presence of BENT-105 on this fragment. We repeated the band shift experiments with restriction fragments from derivatives of plasmid pOSt that carry various deletions of proU 5′ segments (Fig. 2B) (26). In plasmid pA385, part of BENT-105 is deleted, and plasmid pA651 lacks this DNA segment entirely and also does not carry the proU promoter (Fig. 1). Despite the deletions, H-NS still interacted preferentially with the restriction fragments carrying the 5′ segment of the proV gene (Fig. 1). The 454-bp EcoRI-BglI fragment from pA385 that carries proU sequences from -243 to +202 was recognized by H-NS with the highest affinity. At slightly higher H-NS concentrations, we found that also the 192 bp EcoRI-BglI fragment from pA651 (comprising proU sequences from +24 to +202 bp) was efficiently retarded (Fig. 2B). At the same H-NS concentration, a 879-bp restriction fragment carrying the 5′ end of the bla gene was also efficiently bound (Fig. 2B). These restriction fragments are clearly bound by H-NS before the nonspecific binding to any DNA fragment sets in. Therefore, the 192-bp DNA fragment that comprises the beginning of the proV structural gene, but not the proU promoter or the curved upstream sequences, must carry a preferential binding region for H-NS. However, the affinity of H-NS for DNA segments comprising the beginning of the proV structural gene was more pronounced if the fragments included proU sequences located upstream of the proU promoter (Fig. 1). Consequently, these upstream sequences contribute to, but are not required for, specific interactions between H-NS and the 5′ end of the proU operon in vitro.

In a third set of experiments, we analyzed whether the H-NS protein displays any preferential binding directly to the proU promoter itself. Plasmid pBK20 carries a 60-bp proU insert (-54 to +6 bp), which comprises the promoter and the mRNA start site, cloned into the operon fusion vector pMLB1010 (Fig. 3A). We compared the affinity of H-NS to two restriction fragments of 933 bp and 992 bp (derived from pMLB1010 and
A physical map of the proV-lacZ hybrid genes present on plasmids pMLB1010 and pBK20 was integrated as single-copy lysogens at the attB site into the chromosome of K12 strain MKH13 and its isogenic hns-205::Tn10 derivative, JML117. The resulting strains were grown overnight in LBON with the indicated concentrations of NaCl, and the specific p-galactosidase activities of the cells expressed in micromoles of substrate (2-nitrophenyl β-D-galactoside) cleaved/min/mg of protein were determined.

To further corroborate these findings, we analyzed the expression of the proV-lacZ fusion present on plasmid pJL33 in response to the hns-205::Tn10 mutation. Plasmid pJL33 is a derivative of pOS7 in which a copy of an IS1 element had inserted into the spacer region between the proU -10 and -35 sequences (the insertion had occurred between base pairs -23 and -24) (26). This insertion creates a mutant proU promoter composed of the authentic -10 region and a -35 sequence contributed by the IS1 element. The resulting hybrid promoter functions only inefficiently but still permits osmoregulated expression of the proV-lacZ fusion present on the plasmid (26). In plasmid pJL33, all proU sequences upstream of the IS1 insertion point, including the proU -35 region, the curved DNA segment present upstream of the proU operon (BENT-105), and part of the IS1 element are absent. We found that expression of the proV-lacZ fusion of plasmid pJL33 was derepressed in the hns-205::Tn10 mutant (Table I). Therefore, part of the authentic proU promoter and all sequences further upstream can be replaced by a heterologous DNA segment derived from the insertion element IS1 without abolishing the effect of hns mutations on proU expression. Taken together, these experiments show that the repression of proU transcription at low osmolarity by H-NS must depend on DNA sequences located in close vicinity either to the proU -10 promoter region or on sequences present further downstream within the proV gene.

Identification of H-NS Binding Sites in the proU Regulatory Region by DNase I Protection Assays—To identify precisely the binding site(s) of H-NS within H-NS DNA-binding protein H-NS and proU expression.
gene. A number of DNA regions that are protected by the H-NS protein against digestion by DNase I were clearly visible (Fig. 5). Their relative positions on both DNA strands are summarized in Fig. 6. The most extended region protected by H-NS is a 46-bp segment (+64 to +109 bp) which is located at the beginning of the proV structural gene (Fig. 6). In addition to this extended H-NS binding region, we observed a number of additional protected sites that vary in size from 9 to 24 bp (Fig. 5) and encompass a weakly protected region centered around the proU -35 sequence (from -22 to -39 bp) (Fig. 5). We note that H-NS occupation of the extended binding region at the 5' end of proV begins at relatively low protein concentration (0.22 µM), whereas the protection of the region around the proU -35 sequence from DNase I digestion requires a substantially higher H-NS concentration (6.2 µM) (Fig. 5). This finding is consistent with the results from the gel retardation assay (Fig. 3) and shows that the proU promoter itself does not carry a high affinity H-NS binding site (Fig. 3).

We carried out an additional footprinting experiment with a DNA fragment (fragment III) that allowed us to monitor H-NS binding to sequences upstream of the proU promoter. Again, several protected regions with variable size and spacing were visible (Figs. 5 and 6). The H-NS footprints are not regularly distributed along the DNA fragments, indicating that the H-NS molecules are not “phased” in regularly spaced intervals along the DNA. Based on the distinct footprint pattern of H-NS at the proU regulatory region, one can conclude that the H-NS protein does not bind randomly to its substrate, but recognizes specific features of the DNA sequence. We aligned the DNA sequences of the H-NS binding sites from the proU regulatory region (Fig. 6) to look for common sequence determinants, but found no obvious consensus sequence. However, a striking feature of all the DNA segments protected by H-NS is their high A + T content and the presence of uninterrupted stretches of 5 or more A + T base pairs. This is particularly apparent at the extended H-NS binding region at the beginning of the proV gene, where 35 A + T base pairs are found in a 57-bp segment (Fig. 6).

Conformation of the Downstream Binding Site—The H-NS protein has been shown to bind synthetic and naturally occurring curved DNA segments with higher affinity than synthetic non-curved sequences (15, 16). Furthermore, a DNA fragment located downstream of the Salmonella typhimurium proU promoter that is preferentially bound by H-NS displays features of curved DNA (17). We therefore wondered whether the extended H-NS binding site at the 5' end of the E. coli proV gene also consists of curved DNA sequences. We tested several restriction fragments that carry the extended H-NS binding site in proV and different lengths of proU upstream sequences in a two-dimensional gel electrophoresis assay that can resolve DNA fragments according to their size and conformation (Fig. 7). After electrophoresis at 55 °C in the first and 4 °C in the second dimension, “straight” DNA fragments of different size are located on a diagonal line in the gel, whereas curved fragments deviate from the diagonal (59).

The two DNA fragments that extend from position -475 to +202 bp and from -243 to +202 bp within proV (Fig. 7, fragments a and b) and thus contain a large segment of the curved BENT-105 sequence clearly deviated from the diagonal. In contrast, the fragment which extends from -104 to +202 bp and hence comprises the extended H-NS binding region did not show the aberrant mobility expected for a curved DNA fragment (Fig. 7A, fragment d). We repeated this experiment with a restriction fragment extending from position -54 bp to position +370 bp within proV that closely matches the position of a restriction fragment from the S. typhimurium proU locus that has been reported to display features of curved DNA (17). We did not find any significant deviation of this restriction fragment from the E. coli proU promoter region from the normal electrophoretic mobility (data not shown). Therefore, our data do not indicate an overall curved conformation of the extended H-NS binding region at the 5' end of the E. coli proV gene. These experiments cannot rule out that this region might comprise several small bends in different directions that do not add up to a macroscopic curve.

In Vivo Function of the Extended H-NS Binding Region—Our identification of a segment from the 5' end of proV that is bound with high affinity by H-NS in vitro raised the possibility that this region might also mediate the repressing effect of H-NS on proU transcription observed in vivo. To test this hypothesis, we compared the effect of a hns mutation on the expression of two chromosomally proV-lacZ protein fusions that differ in the length of the proV segment but carry identical regions upstream of the proU promoter. The proV-lacZ fusion Δ574-BstNI comprises proV sequences from -54 to +968 bp in proV and thus carries the complete extended H-NS binding region. The proU material in the proV-lacZ fusion Δ574-SspI extends from position -54 bp to position +98 bp and consequently only part of the extended H-NS binding region (+64 to +109 bp) is present in this proV-lacZ hybrid gene (Fig. 8A). In an hns+ background, the Δ574-BstNI proV-lacZ fusion construct carrying the intact extended H-NS binding site displayed the normal regulatory pattern of proU expression: a very low basal level at low osmolarity and a proportional increase of expression when the osmolarity of the medium was raised. In the hns-205::Tn10 mutant, the basal level of expression of this lac fusions was increased about 16-fold and was further stimulated in response to osmotic stress (Fig. 8B).

In comparison with the Δ574-BstNI proV-lacZ fusion, the expression of the Δ574-SspI proV-lacZ construct, in which a part of the extended H-NS binding site was deleted, showed a strongly elevated basal level. This fusion still responded to changes in medium osmolarity with increased expression, which demonstrates in agreement with previous data (17, 51, 60) that proU sequences in the vicinity of the proV promoter are sufficient to mediate a low degree of osmotic regulation. However, a cis-acting DNA segment required to keep the basal level of proU expression low appears to be missing in this construct, which is consistent with several reports describing a “silencer” region within the early part of proV (17, 51, 60). Strikingly, no difference in the level of expression of the Δ574-SspI proV-lacZ fusion construct was detected between the hns+ wild type and the hns-205::Tn10 mutant strain (Fig. 8B). Thus, cis-acting sequence elements required to mediate the repressing effect of the H-NS protein on proU transcription have been affected in this construct.
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DISCUSSION

DNA Binding Properties of H-NS—Mutations in hns (11, 12, 22, 26) or the in vivo sequestration of its gene product by the gene 5.5 protein of bacteriophage T7 (63) result in a derepression of the osmoregulated proU operon at both low and high osmolality. Using purified H-NS protein, we have investigated its interactions with the proU regulatory region by competitive DNA band shift assays and by DNase I footprinting analysis. A preferential binding of H-NS to the proU regulatory region could readily be detected at relatively low H-NS concentration. We found a complex arrangement of H-NS binding sites that differed in extent and varied in spacing along a 420-bp DNA segment surrounding the E. coli proU promoter (Fig. 5). H-NS did not bind to a rigidly defined consensus sequence, but had a marked preference for A+T-rich sequences. All strong H-NS binding sites in the proU regulatory region carried an uninterrupted stretch of 5 or more A+T base pairs (Fig. 6), and we suggest that such A+T tracts might be the main feature of the DNA recognized by H-NS. Interestingly, the binding of H-NS to its substrate can be blocked completely by distamycin, a drug known to interact with the minor groove of A+T stretches in double-stranded DNA (16).

A comparison of the H-NS binding sites detected by us at the proU locus and those previously characterized at the lac and

Fig. 5. DNase I protection assays with H-NS and DNA fragments derived from the proU control region. Three radiolabeled DNA fragments were incubated with the indicated concentrations of H-NS protein, digested with DNase I, and the reaction products were resolved on sequencing gels. Fragment I (proU sequences from -54 to +202 bp) and fragment III (-243 to +202 bp) were labeled at the coding strand; fragment II (-54 to +208) was labeled at the noncoding strand. The lanes labeled A, C, G, and T contain the products of a sequencing reaction used to calibrate the gel and to assign proU sequence coordinates to the products of the DNase I digestion (see "Experimental Procedures"). The position of DNA segments protected against DNase I digestion is indicated by black bars.
gal promoter regions, for which the loosely defined consensus sequence 5'-TNTNAN-3' has been suggested (61), shows a clear but not perfect overlap in sequence specificity. This sequence motif often matches the H-NS footprints observed in the proU regulatory region (Fig. 6). Vice versa, many but not all of the H-NS binding sites found in the lac and gal promoter region include stretches of multiple A.T base pairs (61). When interpreting the H-NS footprinting data for the various promoters, one needs to consider that binding of H-NS to a particular target sequence is concentration dependent (Fig. 3). This is illustrated by the more than 20-fold higher concentration of H-NS required to protect the C-rich 46-bp segment that was protected completely by H-NS in footprinting experiments. The size of the protected region in DNA binding experiments, H-NS shows a clear preference for certain DNA fragments (15-17) (Figs. 2 and 3). The preferentially bound fragment originating from the 5' end of the proV gene analyzed in this study included a A + T-rich 46-bp segment that was protected completely by H-NS in footprinting experiments. The size of the protected region indicates that several H-NS molecules bind to adjacent sites within this segment. The simultaneous occupancy of these sites by H-NS suggests that it binds in a cooperative manner, as has previously been described for the binding of the H-NS protein to the lac promoter region (61). We propose that arrays of several closely spaced stretches of A.T base pairs in an orientation favorable for cooperative interactions of H-NS are a prime determinant for high affinity H-NS binding regions. The intracellular concentration of H-NS in stationary phase cells (5) has been estimated as approximately 18,000 monomers which would correspond to approximately one H-NS dimer (6) per 500 bp of chromosomal DNA. High affinity H-NS binding sites, like the 46-bp region from the 5' end of the proV gene, might serve as nucleation sites to attract additional H-NS molecules through cooperative binding and organize the formation of an extended nucleoprotein complex (64). We note in this context that the DNA segment carrying the hns promoter shows features of curved DNA (20, 21), and that the region around the –10 hexamer of hns is particularly A + T-rich (12, 13). Binding of H-NS to these sequences might explain the negative regulation of hns expression by its own gene product (20, 21). Likewise, H-NS represses the E. coli pap (pili adhesion gene system) promoters located in the highly A + T-rich, intrinsically curved papI-papB intercistronic region (41). We found that H-NS exhibited also a binding preference for restriction fragments carrying the 5' end of the vector-encoded bla gene.

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**Fig. 6. H-NS binding sites in the proU control region.** The DNA sequence of the region analyzed in DNase I protection assays is shown with sequence numbering relative to the transcription initiation site (+1). The segment corresponding to the 5' end of the proV structural gene is presented in capital letters, and the positions of the proU promoter –10 and –35 sequences are indicated. Regions protected by H-NS on the noncoding and coding strand are indicated by black bars, a dotted line represents segments of the DNA strands for which DNase I protection data were obtained only for one DNA strand. Stretches of 5 or more consecutive A.T base pairs are boxed. The occurrence of the sequence motif 5'TNTNAN3' suggested previously (61) as a consensus sequence for H-NS binding is indicated for the noncoding and coding strand with right- and left-pointing arrows, respectively.

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**Fig. 7. Conformational analysis of the extended H-NS binding region by two-dimensional gel electrophoresis.** A. Physical and genetic structure of the (proV-lacZ)hyb2 fusion plasmids pO8T, p3385, and p3524. DNA from these plasmids was mixed and digested with EcoRI and BglII. The origin of the resulting proU restriction fragments is shown schematically; the base-pair numbering is as shown in the upper part of Fig. 1. The letter B indicates a BglII restriction site. The position of the curved BENT-105 DNA segment is shown, and the extended H-NS binding region at the 5' end of the proU gene is marked by a circle. Fragments a, c, and d that comprise this binding region were derived from pO8T, p3385, and p3524, respectively, and differ in the extent of proU sequences present upstream of the proU promoter. B. The mixture of restriction fragments resulting from EcoRI and BglII digests of plasmids pO8T, p3385, and p3524 was resolved on a two-dimensional 4% polyacrylamide gel at 55 °C in the first and at 4 °C in the second dimension, the origin of the gel is at the top left. 1 µg of sheared salmon sperm DNA was added to the restriction fragments before electrophoresis as a marker to indicate the running position of straight DNA fragments on a diagonal across the gel. A negative print of the ethidium bromide stained gel is shown.
The extent of proU sequences is indicated; the insight into the mechanism by which H-NS acts on proU tran-

The topology of the DNA substrate has been implicated in the DNA binding of H-NS, since H-NS displayed a clear preference for curved over noncurved DNA substrates in binding competition experiments (15–17). Such curved DNA segments typically consist of repeated stretches of poly(A) or poly(T), arranged on the same side of the DNA double helix (66). This topological arrangement of A-T stretches might favor cooperative binding of H-NS to such DNA fragments. However, the curved DNA conformation is not a prerequisite for all H-NS binding sites. This view is supported by our analysis of the extended H-NS binding region. Our data do not rule out such a model. However, the H-NS footprint pattern observed by us at the E. coli proU gene. These authors suggest that the H-NS protein interacts with sequences located downstream of the proU promoter (Fig. 8). It was previously shown that a lacZ fusion to base pair +124 of proV that removes most of the proV gene but leaves the 46-bp H-NS binding region intact still reacts to an hns mutation (51). These results suggest that the extended 46-bp H-NS binding region found by us at the 5′ end of proV is a crucial determinant required to mediate the effect of H-NS on proU expression.

How can H-NS protein bound downstream of the promoter affect proU expression? One obvious possibility is that it acts as a transcriptional roadblock and impedes advance of the RNA polymerase. However, in vitro transcription experiments with a proU promoter template showed that H-NS does not affect transcript elongation, but rather early steps in transcription initiation (40). In agreement with the data presented here for the E. coli proU locus, Owen-Hughes et al. (17) recently reported that the H-NS protein interacts with sequences located early in the S. typhimurium proU gene. These authors suggest that the binding of H-NS affects transcription initiation via changes in the local DNA topology that then influence the strength of the proU promoter. Our data do not rule out such a model. However, the H-NS footprint pattern observed by us at the proU regulatory region suggests the presence of an extended nucleoprotein complex that involves sequences both upstream and downstream of the proU promoter (Fig. 6) that would be expected to influence transcription initiation directly, by altering the accessibility of the promoter to RNA polymerase.
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or by influencing the formation of a productive RNA polymerase-promoter complex. This view is supported by the recent report of Ueguchi and Mizuno (40), who demonstrate that H-NS can act as a specific, direct repressor of proU transcription in vitro.

What is the physiological function of H-NS for the expression and regulation of proU? H-NS is clearly not essential for osmotic regulation of proU expression, since hns mutants still show osmotic regulation of proU expression (22). No acting mutations that abolish proU osmoregulation have been found so far. It has therefore been proposed that proU transcription is not regulated by a specific regulatory protein, but by influencing the formation of a productive RNA polymerase-proU promoter complex. This view is supported by the repressing effect of H-NS on proU transcription in vitro. Moreover, sequences in close proximity to the proU promoter, and osmotically relieved repression by high affinity H-NS binding region at the proU promoter, and osmotically relieved repression by high affinity H-NS binding region at the proU promoter are already known in some detail, in which H-NS acts sequence-specific regulatory proteins. We thank M. Haardt for bacterial strains and V. Koogle for help in performing studies presented here suggest that the strong supercoiling of K+-glutamate in vivo. Therefore, the H-NS protein not only serves a role in structuring the chromosomal DNA (18, 23), but also actively participates in the process of gene regulation. Several systems have already been characterized in some detail in which H-NS acts in concert with other, specific regulatory processes and sequence-specific regulatory proteins (28, 30, 32, 37, 38, 41, 42). The active participation of H-NS in many cellular functions thus provides the cell with additional flexibility to fine-tune the level of gene expression in response to demands imposed by the environment.

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