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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* −35 region previously characterized as a target for H-NS binding *in vitro*. We detected a 46-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 (1–3). One of the most abundant nucleoid-associated proteins is H-NS (H1a) (4–7), 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_r = 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 autoregulated, 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 *osmZ* (for an overview, see Ref. 23). Mutations in *hns* lead to changes in gene expression (12, 13, 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, 26). 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 indi-

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rectly 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-galactoside) cleaved/min/mg of protein, was determined as described elsewhere (43).

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 *Δ(putPA)101 Δ(proP)2 Δ(proU)608* mutations which render it entirely deficient in glycine betaine uptake.[1] Strain JML117 is a derivative of MKH13 and carries the *hns-205*::Tn*10* insertion (22). The *bla*$^+$ (*proV'-'lacZ*)(hyb2) protein fusion plasmid pOS7 has been described (43, 53). Plasmids pΔ385, pΔ524, pΔ574, and pΔ651 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 the original *proU* sequence numbering scheme (44, 53) (see Fig. 1). These plasmids carry short stretches of poly(dC·dG) base pairs between a vector-derived *Eco*RI restriction site and the beginning of *proU* sequences, and this is taken into account when we refer to the length of restriction fragments from these plasmids. The specialized transducing phages λpOS7, λpΔ385, and λpΔ574 carry the *proV-lacZ* fusion constructs from plasmids pOS7, pΔ385, and pΔ574, respectively (26).

Plasmid Constructions—Standard methods were used for the construction of recombinant plasmids (54). Plasmid pBK20, which carries a 60-bp[2] *proU* insert (−54 bp to +6 bp; see Fig. 3*A*) in the *lacZ* operon fusion vector pMLB1010 (55), was constructed by excision of the *proU* promoter fragment from pΔ574 with *Eco*RI and *Mae*III. After addition of two base pairs and a *Bam*HI restriction site at the filled-in *Mae*III end through an intermediate cloning step into vector M13BM20 (Boehringer Mannheim), the fragment was ligated into plasmid pMLB1010 that had been cut with *Eco*RI and *Bam*HI. The *proU* promoter of the resulting plasmid, pBK20, reads in the direction of the *lacZ* reporter gene. Plasmid pJL33 is a derivative of the *proU-607*::IS*1* insertion plasmid pJL21 (26), from which all *proU* sequences upstream of the IS*1* insertion point and part of the IS*1* element were removed by digestion with *Eco*RI and *Bst*EII, after which the restriction ends were filled in with Klenow enzyme and religated. The protein fusions vectors pJL28, pJL29, and pJL30, which permit the *in vitro* construction of *lacZ* gene fusions in all three reading frames, carry the multiple cloning sites of plasmids pNM480, pNM481, and pNM482 (56), respectively, in the vector backbone of the protein fusion vector pMLB1034 (55).[3] Plasmids pΔ574-*Ssp*I, pΔ574-*Bal*I, and pΔ574-*Bst*NI carry in-frame *proV-lacZ* protein fusions with different fusion joints. They were constructed by excision of a 0.15-kb *Eco*RI-*Ssp*I fragment, a 0.25-kb *Eco*RI-*Bal*I fragment, and a 0.99-kb *Eco*RI-*Bst*NI fragment from plasmid pΔ574 (Fig. 1) and the insertion of these fragments into the fusion vectors pJL29, pJL30, and pJL28, respectively, that had been opened with *Eco*RI and *Sma*I. The *Bst*NI end of the insert in pΔ574-*Bst*NI was filled in with Klenow enzyme prior to ligation. These plasmid-encoded *proV-lacZ* fusions were transferred by genetic recombination into the *attP*$^+$ specialized transducing phage λRZ5 as previously described (26).

Gel Retardation Assays—The *E. coli* H-NS protein was purified as described by Dersch *et al.* (20). Purified DNA restriction fragments (4 μg) were incubated with H-NS in 20-μl reaction mixtures containing 10 mM Tris-HCl (pH 7.5), 1 mM Na-EDTA, 80 mM NaCl, 10 mM β-mercaptoethanol, and 4% glycerol. After 20 min at room temperature, protein-DNA complexes were resolved on 1.2% agarose gels or 4% polyacrylamide gels with 0.5 × TBE (Tris-borate-EDTA buffer) (54) as the running buffer and stained with ethidium bromide.

DNase I Protection Assays—DNA fragments were asymmetrically labeled by filling up one restriction end with Klenow enzyme in the presence of [^{35}S]dATP (DuPont NEN) and unlabeled nucleotides. Fragment I, comprising *proU* sequences from −54 to +202 bp, was isolated from plasmid pΔ574 (see Fig. 1) by digestion with *Eco*RI and *Bgl*I and labeled upstream of *proU* at the vector-derived *Eco*RI site; after the fill-in reaction it carries 7 bp of vector sequences upstream of *proU*. Fragment II (−54 to +208 bp) was isolated from pΔ574-*Bal*I (Fig. 1) by digestion with *Eco*O109I and *Bam*HI and labeled downstream of the *proU* sequences at the *Bam*HI site; after the labeling it carries 7 bp of vector sequences at the *Bam*HI end and 24 bp at the *Eco*O109I end. Fragment III (−243 to +202 bp) was isolated from pΔ385 (Fig. 1) by digestion with *Eco*RI and *Bgl*I and labeled upstream of the *proU* material at the *Eco*RI site; it carries 11 bp of vector sequences at the *Eco*RI end. DNA fragments (2.5 nM) were incubated with H-NS protein (0.075–6.2 μM) 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 mM Tris-HCl (pH 7.8), 12.5 mM $CaCl_2$, 15 mM $MgCl_2$, 1 mM dithiothreitol, and 200 mM NaCl. The digestion was stopped after 20 s by addition of 50 μl of 15 mM Na-EDTA (pH 8.0) containing 10 μg/ml yeast carrier tRNA. 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).

Two-dimensional Polyacrylamide Electrophoresis—DNA fragments exhibiting a curvature were identified in a mixture of curved and non-curved restriction fragments by a two-dimensional electrophoresis assay (59). The DNA fragments were separated at 55 °C (first dimension) and 4 °C (second dimension) on a 4% polyacrylamide gel with 0.5 × TBE (54) as running buffer and stained with ethidium bromide.

Nucleotide Numbering—*proU* nucleotides in this report are numbered either according to the original reported *proU* sequence numbering (44, 53) (numbers without signs) or relative to the transcriptional start site at position 628 (44, 53) (numbers with +/- sign) (Fig. 1). Positions of restriction sites are given for the first base pair of the recognition sequence, reported extents of restriction fragments refer to the double-stranded segment between the cut sites.

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 pOS7 (Fig. 1). pOS7 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 pOS7 with *Bgl*I 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 to each of these DNA fragments (Fig. 2*A*). At low protein concentrations, H-NS exhibited a preferential affinity for a 680-bp *Bgl*I 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

[1] M. Haardt and E. Bremer, unpublished results.
[2] The abbreviation used is: bp, base pair(s).
[3] J. M. Lucht and E. Bremer, unpublished results.

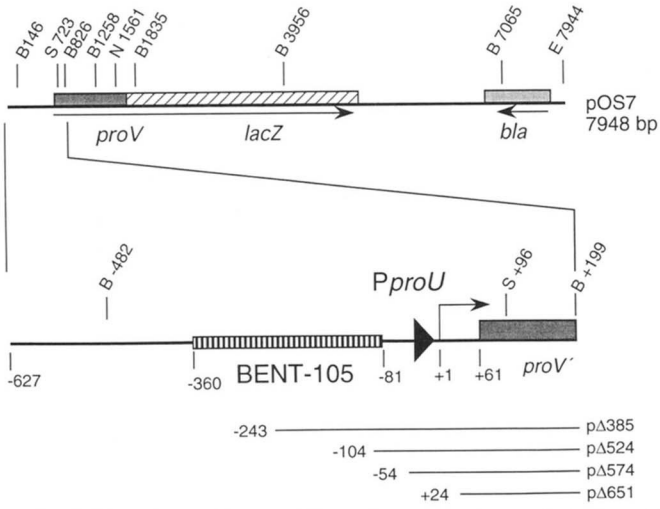

FIG. 1. **Structure of the *proU* regulatory region and of plasmid constructs used in this study.** A map of plasmid pOS7, which carries the complete *proU* regulatory region and a *lacZ* gene fusion to base pair 1582 (+995) of *proV* is shown *above*. The sequence numbering scheme corresponds to that reported by May *et al.* (53). The extended segment of the pOS7 map (*below*) shows the genetic and physical organization of the *proU* regulatory region with a nucleotide numbering relative to the transcriptional start site (*+1*) of the *proU* operon (see "Experimental Procedures"). Restriction sites are abbreviated as: *B*, *Bgl*I; *E*, *Eco*RI; *N*, *Bst*NI; *S*, *Ssp*I. All *Eco*RI and *Bgl*I, but only relevant *Ssp*I and *Bst*NI sites, are shown. At the *bottom*, the DNA segments present in different deletion derivatives of plasmid pOS7 are indicated by *lines*; the extent of the downstream *proU* sequences in these plasmids is identical to that of plasmid pOS7.

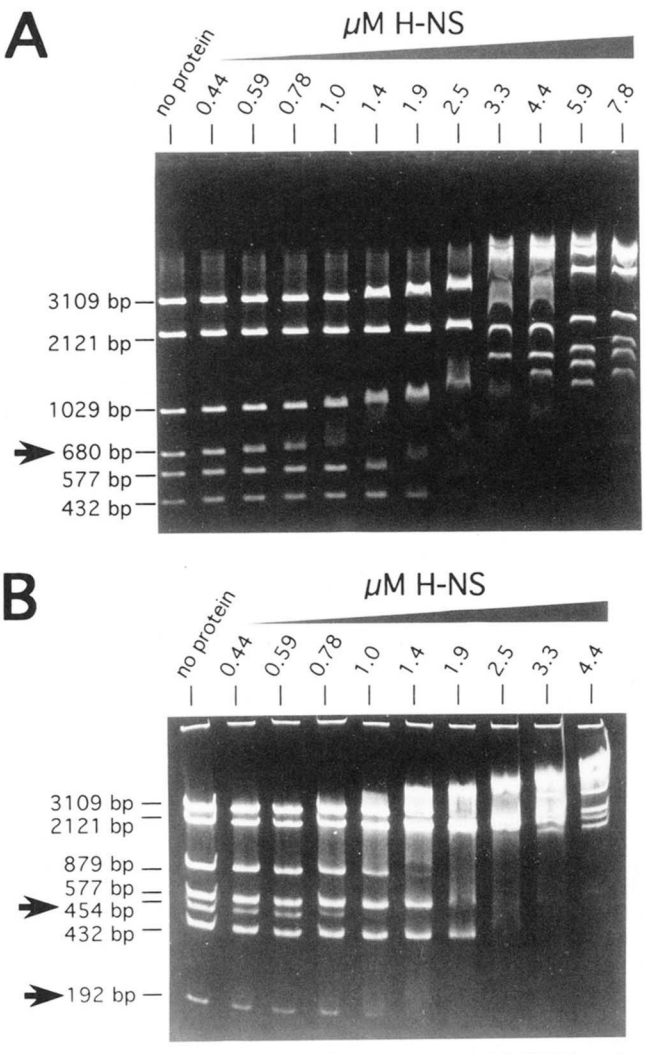

FIG. 2. **Competitive gel retardation assays with H-NS and restriction fragments derived from *proV-lacZ* fusion plasmids.** The relative affinity of H-NS to different DNA fragments was compared. *A*, plasmid pOS7, which carries the complete *proU* control region, was digested with *Bgl*I, the fragments were incubated with the indicated concentrations of H-NS protein, and protein-DNA complexes were resolved by electrophoresis on a 1.2% agarose gel. The *arrow* indicates the position of the 680-bp fragment that carries the *proU* promoter. *B*, plasmids pΔ385 and pΔ651 are derivatives of plasmid pOS7 with different sized deletions of the *proU* upstream sequence. DNA from both plasmids was mixed, cleaved with *Eco*RI and *Bgl*I, and incubated with the indicated concentrations of H-NS. After protein-DNA complex formation, the fragments were resolved on a 4% polyacrylamide gel. The *arrows* mark the position of the fragments that carry the 5′ end of the *proU* operon.

somewhat higher protein concentrations, H-NS also bound efficiently to a 1029- and a 577-bp *Bgl*I fragment (Fig. 2*A*). 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 pOS7, 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. 2*A*). 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 *Bgl*I restriction fragment from the *proU* regulatory region (Fig. 2*A*) was due to the presence of BENT-105 on this fragment. We repeated the band shift experiments with restriction fragments from derivatives of plasmid pOS7 that carry various deletions of *proU* 5′ sequences (Fig. 2*B*) (26). In plasmid pΔ385, part of BENT-105 is deleted, and plasmid pΔ651 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 *Eco*RI-*Bgl*I fragment from pΔ385 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 *Eco*RI-*Bgl*I fragment from pΔ651 (comprising *proU* sequences from +24 to +202 bp) was efficiently retarded (Fig. 2*B*). At the same H-NS concentration, a 879-bp restriction fragment carrying the 5′ end of the *bla* gene was also efficiently bound (Fig. 2*B*). 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. 3*A*). We compared the affinity of H-NS to two restriction fragments of 933 bp and 992 bp (derived from pMLB1010 and

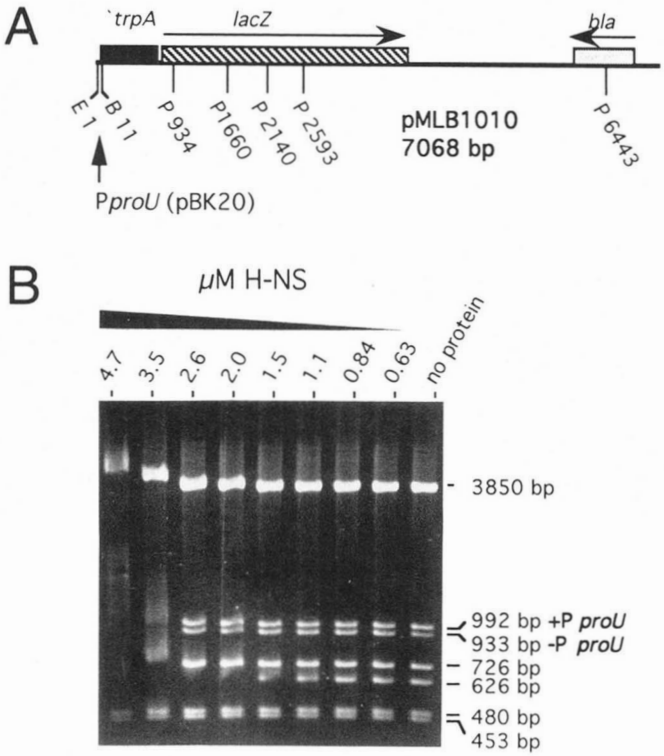

FIG. 3. **H-NS binding to the *proU* promoter.** *A*, physical and genetic maps of the plasmids pMLB1010 and pBK20 used as substrates for the DNA binding assay. The positions and orientations of the *bla*, *lacZ*, and '*trpA* genes of plasmid pMLB1010 are shown, and the position at which the 60-bp *proU* promoter segment has been inserted in plasmid pBK20 is indicated. The positions of restriction sites are given in base pairs. *E*, *Eco*RI; *B*, *Bam*HI; and *P*, *Pvu*I. *B*, competitive gel retardation assay with restriction fragments derived from plasmids pMLB1010 and pBK20. DNA of both plasmids was mixed, digested with *Eco*RI and *Pvu*I, and incubated with the indicated concentrations of H-NS. The reaction mixture was then resolved on a 1.2% agarose gel. The positions of the 933-bp fragment derived from pMLB1010 and the corresponding 992-bp fragment of pBK20 that carries the *proU* promoter are indicated.

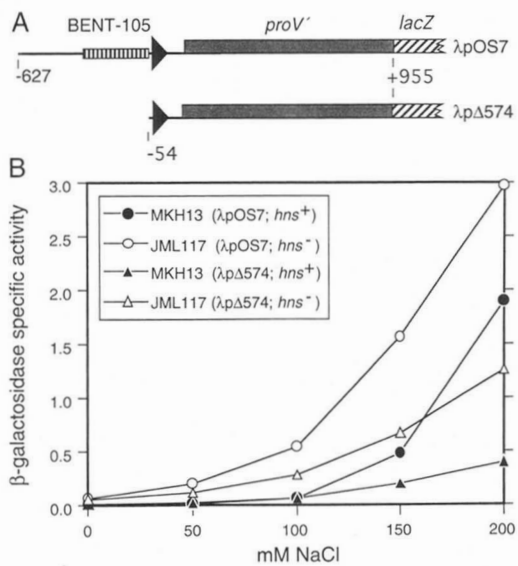

FIG. 4. **Effect of the *hns-205*::Tn*10* mutation on the expression of *proV-lacZ* fusions with *proU* upstream sequences of different length.** *A*, physical map of the *proV-lacZ* hybrid genes present on phages λpOS7 and λpΔ574. The extent of *proU* sequences is indicated, symbols are as in Fig. 1. *B*, phages λpOS7 and λpΔ574 were integrated as single-copy lysogens at the *attB* site into the chromosome of the *hns*$^+$ strain MKH13 and its isogenic *hns-205*::Tn*10* derivative, JML117. The resulting strains were grown overnight in LBON with the indicated concentrations of NaCl, and the specific β-galactosidase activities of the cells expressed in micromoles of substrate (2-nitrophenyl β-D-galactoside) cleaved/min/mg of protein were determined.

pBK20, respectively; Fig. 3*A*) which differ only in the presence of the *proU* promoter segment, in a gel retardation assay (Fig. 3*B*). No retardation of either fragment was observed at low H-NS concentration. The first fragment to be bound by H-NS was a 626-bp restriction fragment which carries the 5′ end of the *bla* gene (Fig. 3). When the H-NS concentration was further increased, both the 933-bp fragment derived from the vector and the 992-bp fragment from pBK20 carrying the *proU* promoter were bound at the same protein concentration. Thus, the H-NS protein displays preferential binding to DNA segments both upstream and downstream of the *proU* promoter, but not to the promoter sequence itself.

Binding of H-NS to Upstream Sequences Does Not Affect proU Expression—DNA sequences located upstream of the *proU* promoter are not involved in osmotic regulation, but their absence reduces the level of *proU* expression *in vivo* (26). These sequences coincide with the curved BENT-105 sequence bound by H-NS *in vitro* (62). We therefore investigated whether the influence of H-NS on *proU* transcription is mediated through its interaction with these upstream sequences. We analyzed the effect of the *hns-205*::Tn*10* mutation on the expression of two chromosomal *proV-lacZ* fusions with upstream regions of different lengths. The fusion present on λpOS7 includes the entire curved BENT-105 DNA segment, whereas λpΔ574 lacks this sequence entirely, since it carries only 19 bp of *proU* material upstream of the −35 hexamer (Fig. 4*A*). Independent of the presence of the curved DNA segment located upstream of the *proU* promoter, the *hns-205*::Tn*10* mutation resulted in a derepression of *proV-lacZ* expression (Fig. 4*B*). Thus, while H-NS interacts with the BENT-105 sequence *in vitro* (62), the influence exerted by the H-NS protein on *proU* expression *in vivo* is clearly not mediated by these curved DNA sequences.

To further corroborate these findings, we analyzed the expression of the *proV-lacZ* fusion present on plasmid pJL33 in response to the *hns-205*::Tn*10* mutation. Plasmid pJL33 is a derivative of pOS7 in which a copy of an IS*1* 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 IS*1* 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 IS*1* insertion point, including the *proU* −35 region, the curved DNA segment present upstream of the *proU* operon (BENT-105), and part of the IS*1* element are absent. We found that expression of the *proV-lacZ* fusion of plasmid pJL33 was derepressed in the *hns-205*::Tn*10* 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 IS*1* 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 sequences located downstream of the *proU* promoter, we performed DNase I protection assays with two DNA fragments (fragments I and II) that comprise the *proU* promoter sequence and include the 5′ end of the *proV*

TABLE I
Influence of the hns-205::Tn10 mutation on the expression of proV-lacZ fusions carrying different DNA segments upstream of the proU-10 regions

Strains MKH13 (*hns*$^+$) or JML117 (*hns-205*::Tn*10*) carrying the *proV-lacZ* fusion plasmid pOS7 or its derivative pJL33, which lacks *proU* sequences upstream of the −10 promoter region, were grown in LBON or LBON with 150 mM NaCl added to increase medium osmolarity. Specific β-galactosidase activity was determined in cells from the mid-logarithmic growth phase.

Strain (plasmid)	β-Galactosidase activity[a]	
	LBON	LBON + 150 mM NaCl
MKH13 (pOS7)	0.25	3.92
JML117 (pOS7)	0.92	11.73
MKH13 (pJL33)	0.22	0.66
JML117 (pJL33)	0.18	1.42

[a] Specific β-galactosidase activity is expressed in micromoles of substrate (2-nitrophenyl β-D-galatoside) cleaved/min/mg of protein.

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 37-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. 7*A*, *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 chromosomal *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-*Bst*NI comprises *proV* sequences from −54 to +936 bp in *proV* and thus carries the complete extended H-NS binding region. The *proU* material in the *proV-lacZ* fusion Δ574-*Ssp*I 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. 8*A*). In an *hns*$^+$ background, the Δ574-*Bst*NI *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*::Tn*10* mutant, the basal level of expression of this *lac* fusion was increased about 16-fold and was further stimulated in response to osmotic stress (Fig. 8*B*).

In comparison with the Δ574-*Bst*NI *proV-lacZ* fusion, the expression of the Δ574-*Ssp*I *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 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-*Ssp*I *proV-lacZ* fusion construct was detected between the *hns*$^+$ wild type and the *hns-205*::Tn*10* mutant strain (Fig. 8*B*). 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.

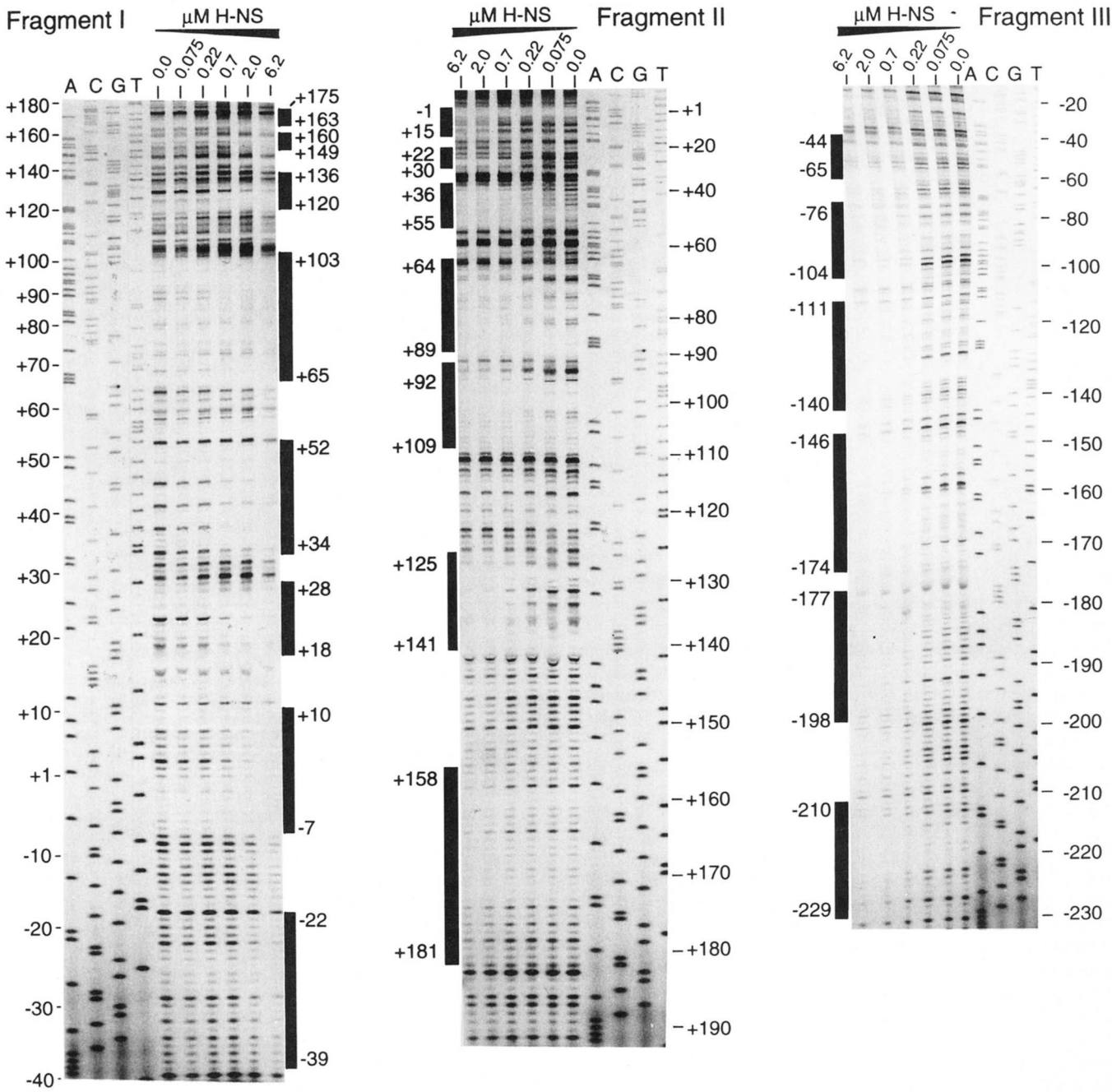

FIG. 5. **DNase I protection assays with H-NS and DNA fragments derived from the *proU* control region.** Three radiolabeled DNA fragments were incubated with the indicated concentrations of H-NS protein, digested with DNase I, and the reaction products were resolved on sequencing gels. *Fragment I* (*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*.

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 osmolarity. 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

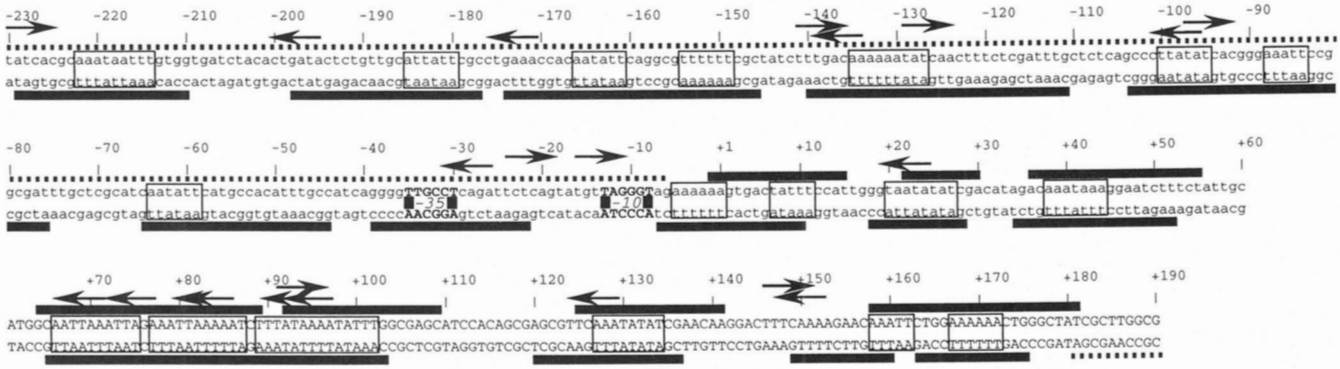

FIG. 6. **H-NS binding sites in the *proU* control region.** The DNA sequence of the region analyzed in DNase I protection assays is shown with sequence numbering relative to the transcription initiation site (*+1*). The segment corresponding to the 5′ end of the *proV* structural gene is presented in *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′-TNTNAN-3′ 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.

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. 5). This is illustrated by the more than 20-fold higher concentration of H-NS required to protect the G + C-rich region around the *proU* −35 sequence (−22 to −39 bp) in comparison to the highly A + T-rich extended H-NS binding region at the beginning of the *proV* gene (+64 to +109 bp) (Fig. 6).

In competitive 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

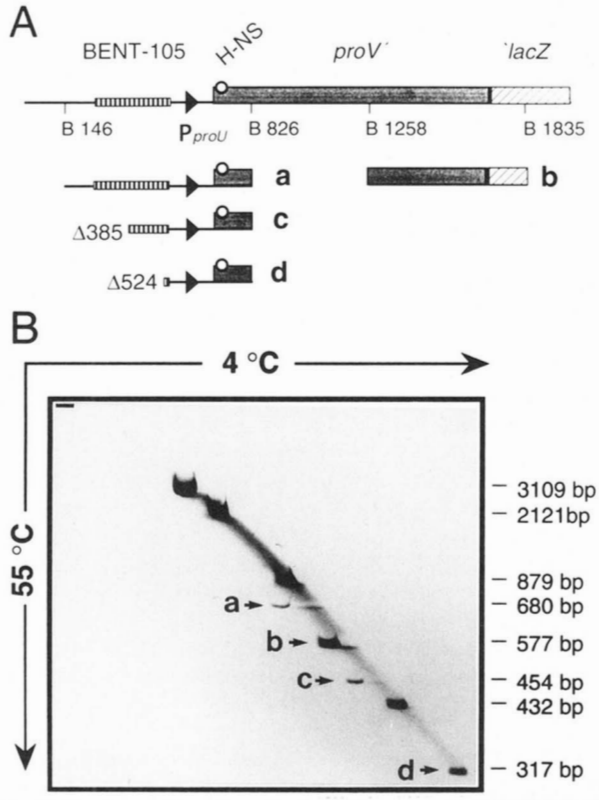

FIG. 7. **Conformational analysis of the extended H-NS binding region by two-dimensional gel electrophoresis.** *A*, physical and genetic structure of the (*proV-lacZ*)(hyb2) fusion plasmids pOS7, pΔ385, and pΔ524. DNA from these plasmids was mixed and digested with *Eco*RI and *Bgl*I. The origin of the resulting *proU* restriction fragments is shown schematically; the base-pair numbering is as shown in the *upper part* of in Fig. 1. The letter *B* indicates a *Bgl*I 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 *proV* gene is marked by a *circle*. *Fragments a*, *c*, and *d* that comprise this binding region were derived from pOS7, pΔ385, and pΔ524, respectively, and differ in the extent of *proU* sequences present upstream of the *proU* promoter. *B*, the mixture of restriction fragments resulting from *Eco*RI and *Bgl*I digests of plasmids pOS7, pΔ385, and pΔ524 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.

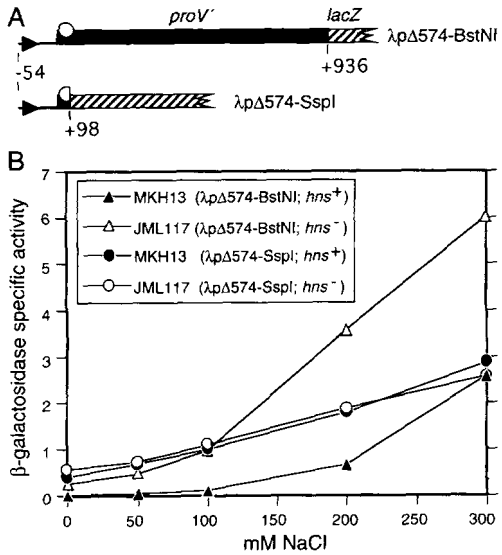

FIG. 8. **Effect of the *hns-205*::Tn*10* mutation on the expression of *proV-lacZ* fusions with *proV* sequences of different length.** *A*, physical map of the *proV-lacZ* hybrid genes present on phages λpΔ574-*Bst*NI and λpΔ574-*Ssp*I. The extent of *proU* sequences is indicated; the *circle* represents the 46-bp H-NS binding site at the 5′ end of *proV* that is partially deleted in λpΔ574-*Ssp*I. The other symbols are as in Fig. 1. *B*, strains MKH13 (*hns*$^+$) and JML117 (*hns-205*::Tn*10*), carrying the prophages λpΔ574-*Bst*NI and λpΔ574-*Ssp*I as single-copy lysogens integrated at the *attB* site, were grown overnight in LBON with the indicated concentrations of NaCl. The specific β-galactosidase activities of the cells expressed in micromoles of substrate (2-nitrophenyl β-D-galactoside) cleaved/min/mg of protein was then determined.

(Figs. 2 and 3), which comprises a curve with a bending center in a highly A + T-rich DNA stretch (29 A·T base pairs in a 33-bp segment) (65).

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 site at the 5′ end of the *proV* gene. DNA fragments comprising this strongly A + T-rich segment are bound by H-NS efficiently (Figs. 2 and 5) but do not show the aberrant electrophoretic mobility expected for curved DNA segments (Fig. 7). We note that the noncurved and curved synthetic DNA substrates used to characterize H-NS as a protein with a preference for curved DNA (15–17) differed strongly in their A + T content. Moreover, the different arrangement of A·T stretches on these fragments might strongly affect the cooperativity of H-NS binding. The observed differential binding of H-NS to curved and noncurved DNA fragments can thus not simply be ascribed to the conformation of these DNA fragments. Additional experiments are required to rigorously define the sequence determinants recognized by the H-NS protein.

Effects of H-NS on Gene Expression—The H-NS protein affects the expression of a wide variety of unrelated genes, and in most cases a negative effect of H-NS on gene expression has been documented (for an overview, see Ref. 23). To gain further insight into the mechanism by which H-NS acts on *proU* transcription, we have correlated its DNA binding to the *proU* promoter region with the effects of *hns* mutations on *proU* expression. Previous experiments have demonstrated the presence of a curved DNA segment upstream of the *proU* −35 region (44) that is recognized efficiently by H-NS *in vitro* (17, 62). However, our deletion analysis of the *proU* upstream region revealed that the negative effect of H-NS on *proU* transcription is not mediated through this curved segment (Fig. 4) but depends on DNA sequences located downstream of the *proU* −35 region (Table I). The different response of two *proV-lacZ* hybrid genes that carried identical upstream sequences including the *proU* promoter but differed in the length of the *proV* segment to the *hns-205*::Tn*10* mutation (Fig. 8) demonstrated that H-NS does not act through a *trans*-acting indirect mechanism targeted at the *proU* promoter sequence, such as a global change in DNA supercoiling. Instead, H-NS requires the presence of *cis*-acting sequences within the *proV* structural gene to negatively affect *proU* expression.

Previous genetic experiments revealed the presence of extended transcriptional silencer sequences at the 5′ end of the *proV* genes from both *E. coli* and *S. typhimurium* that function to keep *proU* expression repressed in medium of low osmolarity (17, 51, 60). Progressive deletions removing parts of this silencer region result in a gradual derepression of *proU* expression. The most pronounced effect is seen when a segment close to the ATG start codon of *proV* is removed (17, 51, 60). Alterations of this *cis*-acting silencer region and *trans*-acting mutations in *hns* have a very similar effect on *proU* expression: both result in a strong increase of the basal level of expression, but do not abolish osmotic control of *proU* transcription. These observations are compatible with a model in which H-NS affects *proU* expression by interaction with the *cis*-acting silencer sequences at the 5′ end of the *proV* gene (17, 60). Indeed, our *in vitro* DNA binding experiments showed that a DNA fragment derived from this region (Fig. 2*B*, bp +24 to +202) was preferentially bound by H-NS in competition experiments, whereas neither the *proU* promoter sequence itself nor two *Bgl*I restriction fragments of 432 and 577 bp that comprise internal *proV* sequences and promoter-distal DNA segments, respectively (Fig. 1), showed a high affinity for H-NS (Figs. 2 and 3). Footprinting experiments revealed the presence of a 46-bp high affinity binding region for H-NS within the preferentially bound fragment (Fig. 6). Part of this site is deleted in the *proV-lacZ* fusion construct Δ574-*Ssp*I that no longer responds to the presence of an *hns* mutation (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 proV* gene. These authors suggest that the binding of H-NS affects *proU* transcription indirectly, 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

or by influencing the formation of a productive RNA polymerase-*proU* 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* transcription, since *hns* mutants still show osmotic regulation of *proU* expression (22). No *trans*-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 changes of the DNA supercoiling (11, 17, 22, 25) or the intracellular ionic composition (40, 67, 68) resulting from changes in the osmolarity of the environment. Such mechanisms are likely to affect transcription initiation directly. Consistent with this, sequences in close proximity to the *proU* promoter have been found to mediate a low degree of osmotic regulation (17, 51, 60) (our data). However, H-NS plays a decisive role in repressing the basal level of *proU* expression in low osmolarity environments, when the components of the ProU transport system are not required by the cell (43, 49). The formation of a nucleoprotein complex by H-NS around the *proU* regulatory region could mediate this repression. Such a structure must be dynamic, since *proU* expression can be rapidly induced by a sudden osmotic upshock (46). Interestingly, it has recently been demonstrated that the repressing effect of H-NS on *proU* transcription *in vitro* is alleviated by a high concentration of K^+-glutamate (40), an ionic condition that mimics that of the cytoplasm of osmotically stressed cells (49). Thus, genetic and biochemical results (40, 51, 69) and the H-NS binding studies presented here suggest that the strong but finely tuned induction of *proU* expression under high osmolarity growth conditions is achieved by a combination of osmotically stimulated transcription, mediated by sequences in the vicinity of the *proU* promoter, and osmotically relieved repression by H-NS, mediated by the high affinity H-NS binding region at the 5′ end of *proV*.

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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REFERENCES

1. Drlica, K., and Rouvière-Yaniv, J. (1987) *Microbiol. Rev.* **51,** 301–319
2. Pettijohn, D. E. (1988) *J. Biol. Chem.* **263,** 12793–12796
3. Schmid, M. B. (1990) *Cell* **63,** 451–453
4. Varshavsky, A. J., Nedospasov, A., Bakayeva, V. V., Bakayeva, T. G., and Georgiev, G. (1977) *Nucleic Acids Res.* **4,** 2725–2745
5. Spassky, A., Rimsky, S., Garreau, H., and Buc, H. (1984) *Nucleic Acids Res.* **12,** 5321–5340
6. Falconi, M., Gualtieri, M. T., La Teana, A., Losso, M. A., and Pon, C. L. (1988) *Mol. Microbiol.* **2,** 323–329
7. Dürrenberger, M., La Teana, A., Citro, G., Venanzi, F., Gualerzi, C. O., and Pon, C. L. (1991) *Res. Microbiol.* **142,** 373–380
8. La Teana, A., Falconi, M., Scarlato, V., Lammi, M., and Pon, C. L. (1989) *FEBS Lett.* **244,** 34–38
9. Hromockyj, A. E., Tucker, S. C., and Maurelli, A. T. (1992) *Mol. Microbiol.* **6,** 2113–2124
10. Marsh, M., and Hillyard, D. R. (1990) *Nucleic Acids Res* **18,** 3397
11. Hulton, C. S. J., Seirafi, A., Hinton, J. C., Sidebotham, J. M., Waddell, L., Pavitt, G. D., Owen-Hughes, T., Spassky, A., Buc, H., and Higgins, C. F. (1990) *Cell* **63,** 631–642
12. May, G., Dersch, P., Haardt, M., Middendorf, A., and Bremer, E. (1990) *Mol. Gen. Genet.* **224,** 81–90
13. Göransson, M., Sondén, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K., and Uhlin, B.-E. (1990) *Nature* **344,** 682–685
14. Friedrich, K., Gualerzi, C. O., Lammi, M., Losso, M. A., and Pon, C. L. (1988) *FEBS Lett.* **229,** 197–202
15. Bracco, L., Kotlarz, D., Kolb, A., Diekmann, S., and Buc, H. (1989) *EMBO J.* **8,** 4289–4296
16. Yamada, H., Muramatsu, S., and Mizuno, T. (1990) *J. Biochem.* **108,** 420–425
17. Owen-Hughes, T. A., Pavitt, G. D., Santos, D. S., Sidebotham, J. M., Hulton, C. S. J., Hinton, J. C. D., and Higgins, C. F. (1992) *Cell* **71,** 255–265
18. Spurio, R., Dürrenberger, M., Falconi, M., La Teana, A., Pon, C. L., and Gualerzi, C. O. (1992) *Mol. Gen. Genet.* **231,** 201–211
19. La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C. L., and Gualerzi, C. O. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10907–10911
20. Dersch, P., Schmidt, K., and Bremer, E. (1993) *Mol. Microbiol.* **8,** 875–889
21. Ueguchi, C., Kakeda, M., and Mizuno, T. (1993) *Mol. Gen. Genet.* **236,** 171–178
22. Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., and Bremer, E. (1988) *Cell* **52,** 569–584
23. Higgins, C. F. (1992) in *Nucleic Acids Mol. Biol.* **6,** 67–81
24. Graeme-Cook, K. A., May, G., Bremer, E., and Higgins, C. F. (1989) *Mol. Microbiol.* **3,** 1287–1294
25. Dorman, C. J., Ni Bhriain, N., and Higgins, C. F. (1990) *Nature* **344,** 789–792
26. Lucht, J. M., and Bremer, E. (1991) *J. Bacteriol.* **173,** 801–809
27. Gutierrez, C., and Devedjian, J. C. (1991) *J. Mol. Biol.* **220,** 959–973
28. Olsén, A., Arnqvist, A., Hammar, M., Sukupolvi, S., and Normark, S. (1993) *Mol. Microbiol.* **7,** 523–536
29. Morschhäuser, J., Uhlin, B.-E., and Hacker, J. (1993) *Mol. Gen. Genet.* **238,** 97–105
30. Shi, X., Waasdorp, B. C., and Bennett, G. N. (1993) *J. Bacteriol.* **175,** 1182–1186
31. Spears, P. A., Schauer, D., and Orndorff, P. E. (1986) *J. Bacteriol.* **168,** 179–185
32. Kawula, T. H., and Orndorff, P. E. (1991) *J. Bacteriol.* **173,** 4116–4123
33. Dri, A. M., Moreau, P. L., and Rouvière-Yaniv, J. (1992) *Gene* **120,** 11–16
34. Yamada, H., Yoshida, T., Tanaka, K., Sasakawa, C., and Mizuno, T. (1991) *Mol. Gen. Genet.* **230,** 332–336
35. Hinton, J. C., Santos, D. S., Seirafi, A., Hulton, C. S., Pavitt, G. D., and Higgins, C. F. (1992) *Mol. Microbiol.* **6,** 2327–2337
36. Lejeune, P., and Danchin, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 360–363
37. Falconi, M., McGovern, V., Gualerzi, C., Hillyard, D., and Higgins, N. P. (1991) *New Biol.* **3,** 615–625
38. Kano, Y., Yasuzawa, K., Tanaka, H., and Imamoto, F. (1993) *Gene* **126,** 93–97
39. Bertin, P., Lejeune, P., Laurent-Winter, C., and Danchin, A. (1990) *Biochim.* **72,** 889–891
40. Ueguchi, C., and Mizuno, T. (1993) *EMBO J.* **12,** 1039–1046
41. Forsman, K., Sondén, B., Göransson, M., and Uhlin, B.-E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 9880–9884
42. Jordi, B. J., Dagberg, B., de Haan, L. A. M., Hamers, A. M., van der Zeijst, B. A. M., Gaastra, W., and Uhlin, B.-E. (1992) *EMBO J.* **11,** 2627–2632
43. May, G., Faatz, E., Villarejo, M., and Bremer, E. (1986) *Mol. Gen. Genet.* **205,** 225–233
44. Gowrishankar, J. (1989) *J. Bacteriol.* **171,** 1923–1931
45. Gowrishankar, J. (1985) *J. Bacteriol.* **164,** 434–445
46. Barron, A., May, G., Bremer, E., and Villarejo, M. (1986) *J. Bacteriol.* **167,** 433–438
47. Cairney, J., Booth, I. R., and Higgins, C. F. (1985) *J. Bacteriol.* **164,** 1224–1232
48. Dunlap, V. J., and Csonka, L. N. (1985) *J. Bacteriol.* **163,** 296–304
49. Csonka, L. N., and Hanson, A. D. (1991) *Annu. Rev. Microbiol.* **45,** 569–606
50. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
51. Dattananda, C. S., Rajkumari, K., and Gowrishankar, J. (1991) *J. Bacteriol.* **173,** 7481–7490
52. Casadaban, M. J. (1976) *J. Mol. Biol.* **104,** 541–555
53. May, G., Faatz, E., Lucht, J. M., Haardt, M., Bolliger, M., and Bremer, E. (1989) *Mol. Microbiol.* **3,** 1521–1531
54. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
55. Silhavy, T. J., Berman, M. L., and Enquist, L. W. (1984) *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
56. Minton, N. P. (1984) *Gene* **31,** 269–273
57. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
58. Yanish-Perron, C., Vieira, J., and Messing, J. (1985) *Gene* **33,** 103–119
59. Mizuno, T. (1987) *Nucleic Acids Res.* **15,** 6827–6841
60. Overdier, D. G., and Csonka, L. N. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3140–3144
61. Rimsky, S., and Spassky, A. (1990) *Biochem.* **29,** 3765–3771
62. Tanaka, K., Muramatsu, S., Yamada, H., and Mizuno, T. (1991) *Mol. Gen. Genet.* **226,** 367–376
63. Liu, Q., and Richardson, C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1761–1765
64. Serrano, M., Salas, M., and Hermoso, J. M. (1993) *Trends Biochem. Sci.* **18,** 202–206
65. Ohyama, T., Nagumo, M., Hirota, Y., and Sakuma, S. (1992) *Nucleic Acids Res.* **20,** 1617–1622
66. Crothers, D. M., Haran, T. E., and Nadeau, J. G. (1990) *J. Biol. Chem.* **265,** 7093–7096
67. Prince, W. S., and Villarejo, M. R. (1990) *J. Biol. Chem.* **265,** 17673–17679
68. Jovanovich, S. B., Record, M. T., Jr., and Burgess, R. R. (1989) *J. Biol. Chem.* **264,** 7821–7825
69. Overdier, D. G., Olson, E. R., Erickson, B. D., Ederer, M. M., and Csonka, L. N. (1989) *J. Bacteriol.* **171,** 4694–4706