# Temperature- and H-NS-Dependent Regulation of a Plasmid-Encoded Virulence Operon Expressing *Escherichia coli* Hemolysin

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Proteins H-NS and Hha form a nucleoprotein complex that modulates expression of the thermoregulated *hly* operon of *Escherichia coli*. We have been able to identify two H-NS binding sites in the *hly* regulatory region. One of them partially overlaps the promoter region (site II), and the other is located about 2 kbp upstream (site I). In contrast, Hha protein did not show any preference for specific sequences. In vitro, temperature influences the affinity of H-NS for a DNA fragment containing both binding sites and H-NS-mediated repression of *hly* operon transcription. Deletion analysis of the *hly* regulatory region confirms the relevance of site I for thermoregulation of this operon. We present a model to explain the temperature-modulated repression of the *hly* operon, based on the experiments reported here and other, preexisting data.

Temperature changes represent signals sensed and processed by the biochemical machinery of the bacterial cell to favor the best possible adaptation and ultimately to allow the microorganism to survive when the environment changes (for a review, see reference 18). A good example of thermosensing is that of many pathogenic bacteria, which sense the temperature increase that they encounter when they enter the human host and respond by triggering the expression of different virulence factors (26). In fact, it is well established that expression of many virulence factors is regulated by environmental parameters, such as temperature, osmolarity, pH, and oxygen availability (11, 12, 27). In spite of the extensive studies carried out in recent years, many molecular aspects of the mechanisms that switch transcription of virulence genes in response to environmental changes remain to be elucidated.

The toxin α-hemolysin (Hly), produced by many uropathogenic *Escherichia coli* strains, is one of the many virulence factors whose expression is thermomodulated (30). The *hly* genes can be either chromosome or plasmid encoded. The *hly* operon harbored by plasmid pHly152 (36), one of the most extensively studied *hly* operons, displays many unusual features. Essential regulatory sequences are spread upstream from the promoter and include a 650-bp sequence, the so-called *hlyR* sequence (42), located more than 1.5 kbp upstream of *hlyC*, the first structural gene of the operon. An antiterminator element (the *ops* element), frequently found in long bacterial operons such as those encoding lipopolysaccharides and capsule synthesis or fertility, is located within the *hlyR* sequence (32). Furthermore, an IS2 insertion element separates *hlyR* from the promoter region (Fig. 1). Deletion of *hlyR* results in an unnatural repression of hemolysin expression (4, 42). This phenotype could be interpreted as being caused by the loss of the above mentioned *ops* element, but this has not

yet been demonstrated. In addition to this as yet unclear antitermination mechanism, the *hly* operon is regulated by both temperature and osmolarity (29, 30). Studies focused on the regulatory features of the *hly* operon of plasmid pHly152 led to the identification of the Hha protein (4, 33) and to the finding that *hha* mutants are partially derepressed under conditions involving temperature- and osmolarity-mediated hemolysin repression. The Hha protein belongs to a new family of modulators, which includes, among others, the YmoA protein, a temperature-dependent modulator of the expression of different virulence factors in *Yersinia enterocolitica* (5). Both proteins display a high degree of conservation, with 82% of their amino acid sequences identical, and are functionally interchangeable (2, 7, 28).

Recently it has been found that Hha interacts with the nucleoid-associated protein H-NS (35). H-NS, a major component of the bacterial nucleoid, plays a central role as a modulator of gene expression in response to osmolarity and temperature changes (for reviews, see references 1, 41, and 43). In fact, H-NS, which binds preferentially to curved DNA (44) and is able to generate bends in noncurved DNA (38), affects the expression of a large number of genes (1, 16, 24) and is considered a general negative transcriptional regulator. Different models have been suggested to explain the mechanism by which H-NS represses gene expression. According to one model, termed "transcriptional silencing" (14), H-NS binding to DNA would occlude RNA polymerase access through a generalized effect on DNA compaction. Another model includes a direct competition between H-NS and the RNA polymerase for overlapping binding sites on or near the promoter (10, 40). Alternatively, H-NS would exert its repression through modifications of DNA supercoiling (17, 20, 39).

Interaction of H-NS with proteins other than Hha has been previously reported, but the significance of such interactions remains unclear (8, 22, 25, 31). In contrast, the phenotype of the *hha/hns* double mutants, which have lost both thermo- and osmoregulation (35), indicates a clear role of the Hha–H-NS complex in the thermo-osmotic regulation of the *hly* operon.

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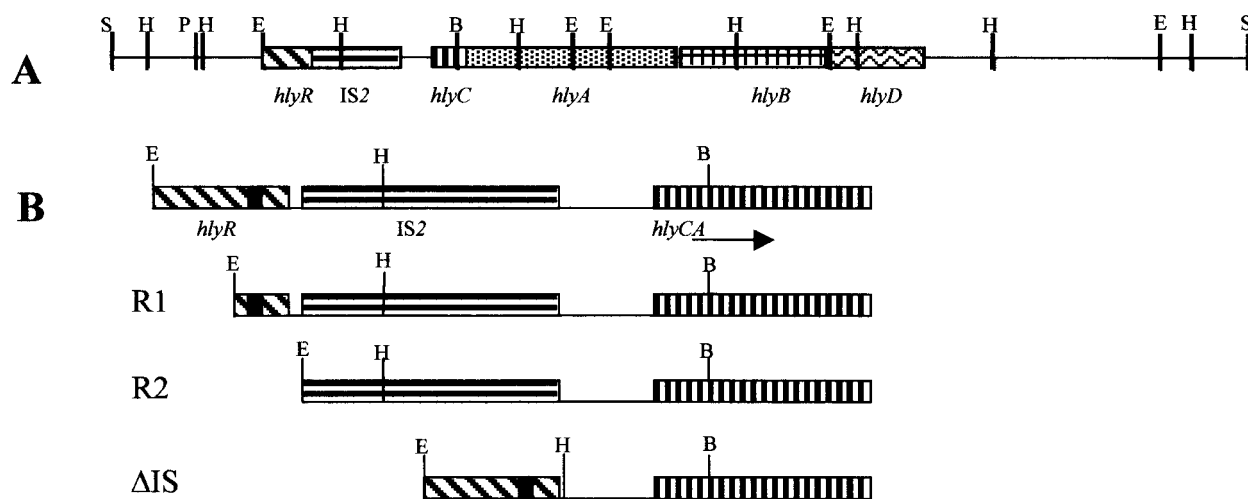
FIG. 1. Schematic representation of the upstream and coding regions of the *hly* operon of plasmid pHly152. The *hlyR* sequence, the insertion element IS*2*, and sequences corresponding to the structural genes of the operon (*hlyC, hlyA, hlyB, and hlyD*) are indicated as bars with different patterns. The black box, in part B, indicates the *ops* sequence. E, H, S, P, and B correspond to restriction sites for *Eco*RI, *Hin*dIII, *Sal*I, *Pst*I, and *Bam*HI, respectively. R1, R2, and ΔIS correspond to the deletions generated in this study.

For this work, we have further investigated the mechanism underlying Hha- and H-NS-mediated thermoregulation of the *hly* operon. We show that, rather than Hha, H-NS is the protein that binds at specific DNA sequences. In addition, we show that temperature is a critical factor in the modulation of the H-NS affinity for two distant DNA sequences of the regulatory region of the *hly* operon. A delicate balance between the moderate affinity of H-NS for its target sequences in this region and temperature-mediated changes in DNA flexibility is proposed as the factor allowing H-NS to generate a nucleoprotein complex (including Hha as well) that renders the *hly* operon repressed at low temperature.

### MATERIALS AND METHODS

**Bacterial strains, plasmids and media.** *E. coli* strains 5K (21), BSN26 (*hha*[+] *hns*[+]) (19), BSN26H (*hha hns*[+]) (35), BSN27 (*hha*[+] Δ*hns*) (19), and BSN27H (*hha* Δ*hns*) (35) and plasmids pHly152 (36) and pANN202312-R (13) have been previously described.

Plasmids pLGHlyR0, pLGHlyR1, pLGHlyR2, and pLGHlyΔIS are pLG338-30 (6) derivatives. Plasmid pLGHlyR0 was constructed by cloning the complete *hly* operon of plasmid pHly152, from the *Eco*RI site upstream of the *hlyR* sequence to the *Sal*I site downstream of the *hlyD* sequence (Fig. 1). To do this, the *Eco*RI-*Bam*HI fragment corresponding to the regulatory region of the *hly* operon was first cloned into pLG338-30, generating pLGR0. Upon *Bam*HI-*Sal*I digestion, the 11.7-kbp *Bam*HI-*Sal*I fragment of the *hly* operon from plasmid pHly152 was inserted, generating pLGHlyR0.

A similar strategy was used to construct plasmids pLGHlyR1, pLGHlyR2, and pLGHlyΔIS, which contain different deletions of the regulatory region of the *hly* operon (Fig. 1). The *Eco*RI-*Bam*HI fragment of this plasmid was first modified to generate the deletions that were synthesized by PCR, using the primers described in Table 1. Primers HlyR1/HlyBam and HlyR2/HlyBam, containing *Eco*RI and *Bam*HI restriction sites, were used to amplify the different *Eco*RI-*Bam*HI fragments that were cloned into pLG338-30, generating plasmids pLGR1 and pLGR2, respectively. To construct the *Eco*RI-*Bam*HI fragment containing the deletion of the IS*2* element, we used primers HlyR0/ISA, with *Eco*RI and *Hin*dIII restriction sites, and ISB/HlyBam, with *Hin*dIII and *Bam*HI restriction sites. These fragments were digested with *Hin*dIII, ligated, and then cloned as *Eco*RI-*Bam*HI fragments into pLG338-30, obtaining plasmid pLGΔIS. We reconstructed the *hly* operon in all these plasmids by cloning the 11.7-kbp *Bam*HI-*Sal*I fragment of the *hly* operon of plasmid pHly152 to generate plasmids pLGHlyR1, pLGHlyR2, and pLGHlyΔIS.

**Gel retardation assays.** Binding of H-NS to DNA was carried out in 20 μl of 40 mM Tris-HCl (pH 8.0)–100 mM KCl–1 mM dithiothreitol (DTT)–5 mM K phosphate–5% glycerol–50 mM EDTA, containing increasing concentrations of purified H-NS (0.12 to 0.48 μM) and 50 ng of DNA. Either linear DNA corresponding to the *Eco*RI-*Bam*HI fragment of plasmid pLGR0 or the supercoiled DNA corresponding to plasmid pLGR0 was used. After incubation for 20 min at 25 or 37°C, the protein-DNA complexes were subjected to electrophoretic separation at 100 V at constant temperature (25 or 37°C) in 0.8% agarose gels in 0.5× Tris-borate-EDTA buffer. The DNA was visualized by ethidium bromide stain.

**Gel competition retardation assay.** The DNA fragments used in this assay were obtained by digesting the *Sal*I-*Bam*HI fragment of the *hly* operon (Fig. 2). This fragment was first *Eco*RI digested, and the two resulting fragments were then purified. The *Sal*I-*Eco*RI fragment was subsequently cut with *Hin*dIII and *Pst*I, thus generating fragments of 0.1, 0.5, 0.7, and 0.9 kbp. The band corresponding to the 0.1-kbp *Pst*I-*Hin*dIII fragment was not detected under the electrophoretic conditions used. The *Eco*RI-*Bam*HI fragment was *Hin*dIII digested, generating fragments of 1.1 and 1.7 kbp. All these fragments were mixed either with Hha (19.12 to 76.48 μM) or with H-NS (0.24 to 0.97 μM) and incubated 10 min at 37°C under the conditions described above and then loaded in 1.5% agarose gels. Electrophoresis was carried out at room temperature, and the DNA was visualized by ethidium bromide staining.

**In vitro transcription assay.** Transcription reactions containing 150 ng of supercoiled plasmid DNA (pANN202-312-R) and 1 U of *E. coli* RNA polymerase (USB) were performed in 40 mM Tris-HCl (pH 8.0)–10 mM MgCl₂–5 mM DTT–200 μM ATP, CTP, GTP, and UTP–4 U of RNasin (Promega)–50 ng of BSA–150 mM KCl. The reaction mixtures were incubated for 30 min at 25°C or 37°C, with or without increasing amounts of H-NS protein (0.39 to 1.17 μM). The

TABLE 1. Oligonucleotides used in this study to amplify DNA fragments corresponding to different deletions (HlyR1 and HlyBam to amplify the R1 fragment, HlyR2 and HlyBam to amplify the R2 fragment, and HlyRO/ISA and ISB/HlyBam to amplify the fragments corresponding to ΔIS)

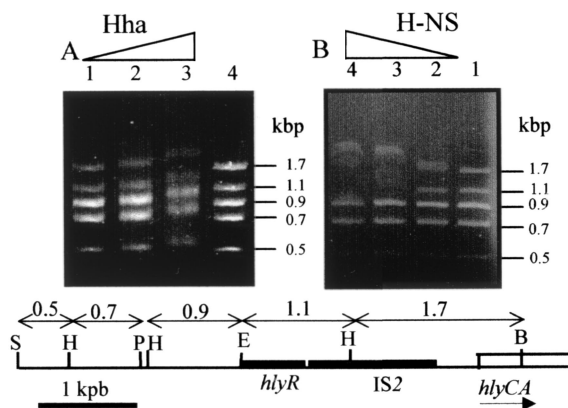| Oligonucleotide | Sequence |
|---|---|
| HlyRO | 5′-GGGGAATTCCAAGCGAAGTCCA-3′ |
| HlyBam | 5′-GTTTTGGGGATCCACCCTGATGG-3′ |
| HlyR1 | 5′-ACTCCTTCAGAATTCTGGTCCC-3′ |
| HlyR2 | 5′-GGGAATTCTCCAGACAACCAA-3′ |
| ISA | 5′-ATCTCATAAAGCTTGATGC-3′ |
| ISB | 5′-TATCTGAAGCTTGACGAAGT-5′ |

FIG. 2. Effect of increasing concentrations of Hha (A) and H-NS (B) proteins on the electrophoretic mobility of DNA fragments from the regulatory region of the *hly* operon. These fragments and their sizes are shown in the lower part of the figure, where the sequences corresponding to *hlyR* and IS*2* (black boxes) and to the *hlyC* and *hlyA* genes (white boxes) and the restriction sites for *Sal*I (S), *Pst*I (P), *Hin*dIII (H), *Eco*RI (E), and *Bam*HI (B) are indicated. Lanes 1, 2, 3, and 4 of panel A contained 19.12, 38.24, 76.48, and 0 μM Hha protein, respectively. Lanes 1, 2, 3 and 4 of panel B contained 0, 0.24, 0.48 and 0.97 μM H-NS protein, respectively.

reactions were stopped by the addition of 1 μl of 0.5 M EDTA, 10 μg of carrier tRNA, 3 μl of 3 M sodium acetate (pH 5.4), and 90 μl of ethanol. RNA was precipitated and analyzed by primer extension.

**Primer extension of RNAs.** The RNA was resuspended in a solution containing 40 mM Tris-HCl (pH 7.5)–20 mM MgCl$_2$–50 mM NaCl–8 U of RNasin–5 pmol of the oligonucleotide used (5′-AACTTAAATTATATGATTAAA-3′), which hybridizes coordinates 2466 to 2486 (see Fig. 7). The mixture was heated for 5 min at 90°C and then allowed to cool slowly to 20°C. The solution was then put on ice for 5 min, and the RNA was precipitated by the addition of a 1/10 volume of 3 M sodium acetate (pH 5.4) and 2.5 volumes of ethanol. Precipitated RNA was resuspended in 5 μl of water, and the primer was extended in a solution containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl$_2$, 0.5 mM spermidine, 10 mM DTT, 8 U of RNasin (Promega), 0.2 mM each dNTP (except dATP, which was 100 μM), 2 μCi of [α-$^{32}$P]dATP (3,000 Ci/mmol; Amersham Pharmacia Biotech), and 10 U of AMV-reverse transcriptase (Promega) in a total volume of 10 μl.

The reaction mixture was incubated at 42°C for 45 min and then stopped by addition of 0.5 μl of 0.5 M EDTA and 30 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. The sample was filtered through a 1-ml Sephadex G-50 spin column to eliminate the nonincorporated labeled nucleotide. The eluted cDNA was precipitated by the addition of 4 μl of 3 M sodium acetate (pH 5.4) and 200 μl of ethanol. The cDNA was analyzed in urea–6% polyacrylamide gel electrophoresis (PAGE) gels and visualized by autoradiography.

**Measurement of *hly* operon transcription by RNase protection assay.** Total cellular RNA was isolated from mid-exponential-phase cultures (optical density at 600 nm = 0.5) using the acid phenol method (23). Plasmid pGEMhlyC (33) contains a fragment of the *hlyC* gene under the control of the T7 RNA polymerase promoter. Linearized (*Nde*I) pGEMhlyC was used as template to obtain antisense radiolabeled probes to the *hlyC* gene by in vitro transcription with T7 RNA polymerase in the presence of [α-$^{32}$P]UTP. Purity of the probe was checked by urea–6% PAGE.

For the RNase protection assay, 25 μg of total RNA was hybridized to an excess of radiolabeled probe and the nonhybridized RNA and probe were degraded with RNase-ONE (Promega). The protected probe was separated in urea–6% PAGE gels and visualized by autoradiography.

**DNase I footprinting.** Supercoiled plasmid pLGHlyR0 (100 ng per sample) was preincubated for 20 min at 25°C with the appropriate concentrations of purified H-NS. After DNase I treatment, the partial-digestion DNA products were ethanol precipitated and subjected to 30 cycles of linear PCR using 5′-end $^{32}$P-labeled primer and *Taq*I polymerase (Polymed) essentially as previously described (11). The extension PCR products were separated on a 7% sequencing gel. The convergent oligonucleotide pair Hly543 (5′-CCAAGCGAAGTCCAT

CCCCCTCC-3′ [coordinates 1 to 28]) and Hly544 (5′-CTTGAAGGAGTGAG TTTGGATATGG-3′ [coordinates 473 to 448]) and the convergent oligonucleotide pair Hly545 (5′-CCTGGTCATTATCTGGAATTTGACG-3′ [coordinates 2032 to 2056]) and Hly546 (5′-CCCATAGCCAGGATACATGCCC-3′ [coordinates 2552 to 2531]) were used to detect H-NS protection on both DNA strands at sites I and II, respectively.

**Atomic force microscopy.** Samples were prepared by placing a drop (2 μl) of DNA solution onto freshly cleaved green mica (Ashville-Schoonmaker Mica Co., Newport News, Va.). After adsorption for 5 min at room temperature, the samples were rinsed for 10 s in a jet of deionized water of 18 MΩ/cm from a Milli-Q water purification system (Millipore, Molsheim, France), directed onto the surface with a squeeze bottle. The samples were dried in an isolated chamber under pure N$_2$ pressure for 30 min before imaging in the atomic force microscope (AFM) (37).

The samples were imaged in a Nanoscope III Multimode AFM (Digital Instruments Inc., Santa Barbara, Calif.), operating in tapping mode in air (15), at a scan rate of 1 to 2 Hz. The AFM probes were 125-μm-long monocrystal silicon cantilevers with integrated conical Si tips (Nanosensors GmbH, Norderfriedrichskoog, Germany) with an average resonance frequency of ≈300 kHz and a spring constant K of ≈35 N/m. The cantilever is rectangular, with a tip radius as given by the supplier of 5 to 15 nm, a cone angle of 35°, and a high aspect ratio. In general, the images were obtained at room temperature (23 ± 2°C) and the relative humidity was typically 55%.

**Preparation of H-NS and Hha proteins.** Hha and H-NS proteins were purified as described previously (see references 9 and 35, respectively).

**Curvature predictions.** Computer-generated predictions of intrinsic curvature were obtained with 3D-WEDGE, a program developed by G. Micheli (11).

**Nucleotide sequence accession numbers.** Accession numbers of DNA sequences (all previously published) used in this work are x07565 (*hlyR* sequence), m14107 (plasmid pHly152 *hlyC*, *A*, *B* and *D* genes), and m18426 (insertion element IS*2*). These sequences are partially overlapping and were used to create a composite of the upstream regulatory region of the *hly* operon.

## RESULTS

**Interaction between H-NS and the regulatory region of the *hly* operon.** We had previously tested binding of Hha and H-NS proteins to different DNA fragments of the *hly* regulatory region in the presence of competitor DNA. This experimental approach did not allow us to determine preferred binding sites for either protein (35). To find such sites, we have used a different approach: we performed competition gel retardation assays, selecting a mixture of DNA fragments corresponding to the *hly* regulatory region and increasing concentrations of either Hha or H-NS. As seen from Fig. 1 and the lower part of Fig. 2, the *Sal*I-*Bam*HI restriction fragment of plasmid pHly152 spans approximately 4.9 kb upstream of the *Bam*HI site located at *hlyC*, the first structural gene of the *hly* operon. This fragment includes the 2.8-kbp region (the 1.1-kbp *Eco*RI-*Hin*dIII fragment plus the 1.7-kbp *Hin*dIII-*Bam*HI fragment) that should contain all the regulatory elements controlling the expression of this operon. To study the interaction between Hha and H-NS proteins and this DNA region, competition gel retardation assays were performed using a mixture of five DNA restriction fragments derived from the *Sal*I-*Bam*HI fragment (Fig. 2). The mixture was incubated in the presence of increasing concentrations of either Hha or H-NS. We found that the Hha protein does not display a preferential binding for any one of the five DNA fragments present in the reaction mixture (Fig. 2A). In contrast, H-NS exhibits a preferential interaction with the two fragments (the 1.7- and 1.1-kbp fragments) that contain predicted regulatory sequences (Fig. 2B). The fact that both fragments are preferentially retarded by H-NS suggested that there is more than one binding site for this protein within the regulatory sequences of the *hly* operon. Furthermore, since H-NS participates in the thermoregulation
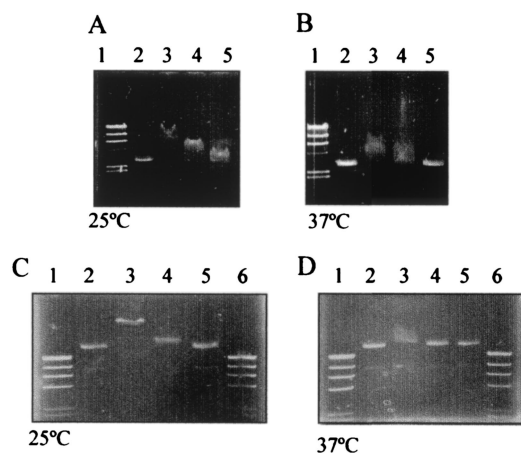
FIG. 3. Effect of increasing concentrations of H-NS on the electrophoretic mobility of the linear 2.8-kbp *Eco*RI-*Bam*HI DNA fragment derived from the regulatory region of the *hly* operon (Fig. 2) (A and B) and on the mobility of plasmid pLGR0 (C and D). Binding reactions and electrophoretic separations were performed either at 25°C (A and C) or at 37°C (B and D). (A and B) Lane 1, molecular weight markers; lane 2, no protein added; lanes 3 to 5, 0.48, 0.24, and 0.12 μM H-NS protein, respectively. (C and D) Lanes 1 and 6, molecular weight markers; lane 2, no protein added; lanes 3 to 5, 0.97, 0.48, and 0.24 μM H-NS protein, respectively.
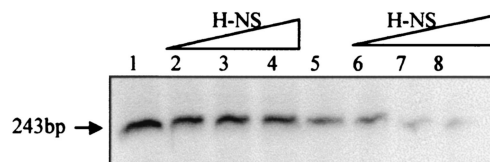


FIG. 4. Primer extension assay of the *hly* operon in the presence or absence of H-NS protein at 37°C (lanes 1 to 4) and at 25°C (lanes 5 to 8). Lanes 1 and 5, no protein added; lanes 2 and 6, 0.39 μM H-NS; lanes 3 and 7, 0.78 μM H-NS; lanes 4 and 8, 1.17 μM H-NS. Further experimental details are given in Materials and Methods.

of the *hly* operon expression, it could be envisaged that the interaction of H-NS with these sites could be modulated by temperature within the physiologically relevant range.

**Temperature affects the H-NS affinity for the 2.8-kbp *hly* regulatory region.** To determine whether the interaction of H-NS with the *hly* regulatory sequences could be affected by temperature, H-NS binding experiments were performed. Increasing concentrations of H-NS were mixed and incubated with the complete 2.8-kb *Eco*RI-*Bam*HI linear DNA fragment at constant temperature (either 25°C or 37°C), and the interaction was determined by gel retardation assays, performed at the same temperature. The results obtained clearly indicate that H-NS shows more in vitro affinity to the DNA fragment at 25°C (Fig. 3A) than at 37°C (Fig. 3B). To investigate this temperature effect further, we performed experiments similar to those described above, replacing the linear DNA with supercoiled DNA. To this end, plasmid pLGR0, which contains the *Eco*RI-*Bam*HI DNA fragment, was used to test the effect of temperature on H-NS affinity for the *hly* regulatory region. When pLGR0 was incubated in the presence of increasing concentrations of H-NS and the resulting protein-DNA complexes were separated in 0.8% agarose gels under conditions of constant temperature (either 25°C or 37°C), the results obtained clearly showed that the interaction of H-NS with pLGR0 is influenced by temperature and that the affinity is significantly higher at 25°C (Fig. 3C) than at 37°C (Fig. 3D). Taken together, the results determined so far indicate that unlike Hha, H-NS is able to select and bind specific regulatory sequences with the upstream region of the *hly* operon and that, at least in vitro, this binding is temperature dependent. Furthermore, taking into account the occurrence of an interaction between Hha and H-NS, these findings suggest that H-NS is the protein responsible for selecting and binding to specific DNA sites in the *hly* operon and that it thereby induces the

temperature-dependent formation of a nucleoprotein complex, also containing Hha, that is responsible for the transcriptional repression of this operon.

**Temperature-mediated repression of *hly* operon transcription by H-NS.** When analyzing the in vivo effect of an *hns* allele, we found that H-NS protein plays a major role in repressing hemolysin expression in *E. coli* cells growing at low temperature (35). In order to establish a correlation between the higher affinity that H-NS shows at 25°C for regulatory sequences of the *hly* operon (Fig. 3) and the observed role of H-NS in repressing transcription, we have analyzed the effect of H-NS on the in vitro transcription of the *hly* operon at different temperatures. Previous results indicated that transcription of the *hly* operon from plasmid pANN202-312R is more efficient when supercoiled DNA is used as a template (C. Madrid, unpublished data). Thus, supercoiled plasmid pANN202-312R was used as the template in transcriptional assays carried out at either 25 or 37°C in the presence of increasing amounts of purified H-NS. The data obtained (Fig. 4) indicate that H-NS-mediated repression of the *hly* operon is temperature dependent. We have quantified the results (using Molecular Analyst software by Bio-Rad) to determine the reduction in transcription levels that the presence of H-NS protein causes. At 37°C, the highest concentration of H-NS used accounts for a 20% reduction of transcription, whereas at 25°C, the reduction is about 60%. The finding that H-NS represses transcription of the *hly* operon more efficiently at 25°C than at 37°C correlates well with the fact that H-NS shows more affinity for regulatory sequences of the *hly* operon at low temperature and with the observed phenotype of *hns* mutants, which derepress hemolysin expression at low temperature (35).

**Identification of H-NS binding sites by footprinting.** The above results prompted us to map the H-NS binding sites on the *hly* regulatory region by performing in vitro DNase I footprinting at 25°C in the presence and absence of this protein. These experiments were carried out on a supercoiled pLGHlyR0 plasmid carrying the entire *hly* operon, from the *Eco*RI site just upstream of *hlyR* to the *Sal*I site downstream of *hlyD* (Fig. 1A). As seen from the results presented in Fig. 5A and B, H-NS influences the accessibility to DNase I digestion of two fairly extended sites, identified as site I and site II, for which H-NS seems to have, within the limits of accuracy of this type of analysis, a comparable affinity. Site I, which spans nucleotides 214 to 358 on one filament and nucleotides 193 to 348 on the other (Fig. 5A and B), clearly overlaps the *hlyR* sequence. Site II, on the other hand, extends approximately from nucleotide 2184 to 2285 on one filament and from nucleotide 2215
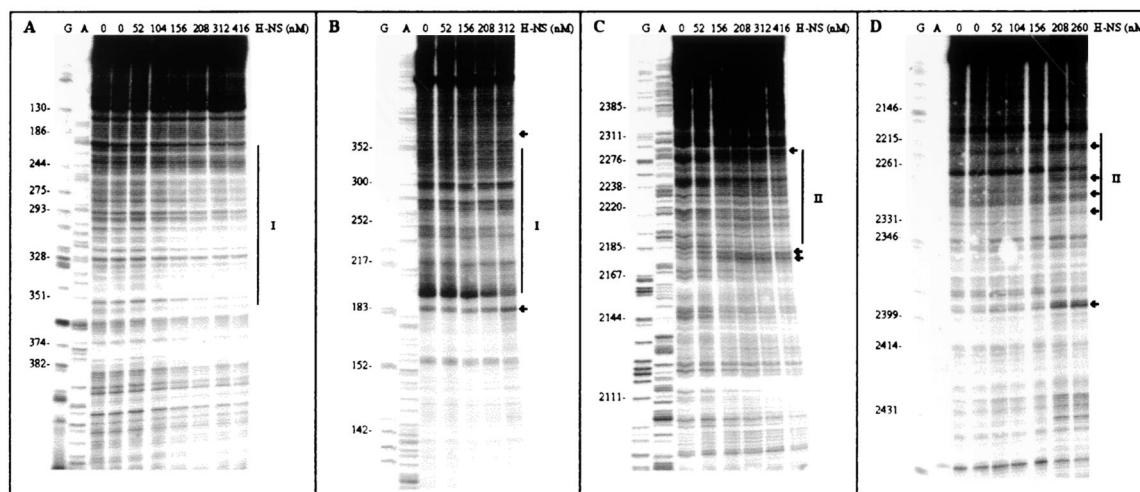
FIG. 5. DNase I footprinting of *hlyR* regulatory region by H-NS. Plasmid pLGHlyR0 was incubated at 25°C with or without the amounts of H-NS (expressed as nanomolar concentrations of dimers) indicated above each lane. The samples were processed using Hly544 (A), Hly543 (B), Hly545 (C), and Hly546 (D) as primers, as described in Materials and Methods. Lanes G and A represent *Taq*I polymerase sequencing reactions using the same primers. The H-NS-protected regions are indicated with vertical lines and labeled I and II; the sites hypersensitive to DNase I are indicated by arrows.

to 2329 on the other (Fig. 5B and C) and overlaps two of the three promoters described for the *hly* operon of plasmid pHly152 (23) (Fig. 6). Thus, site I and site II are flanking the IS2 insertion element such that the intrinsic DNA curvature predicted to occur within the IS2 element (Fig. 7A) can favor the interaction between the H-NS molecules located at both sites and thereby produce a stable H-NS-DNA complex. The hypothesis of the actual existence of the computer-predicted curvature is supported by an analysis of the *Eco*RI-*Bam*HI fragment carried out by AFM, which revealed the presence of a strongly curved region approximately located in the central region of this fragment (Fig. 7B). This conclusion is also supported by additional data obtained by gel electrophoresis in agarose medium, which shows different levels of mobility of this particular *Eco*RI-*Bam*HI fragment at 55°C and 4°C (our unpublished data). In addition to this intrinsic DNA bend, the appearance of several points (indicated by the arrows in Fig. 5) where H-NS induces an enhanced sensibility to DNase I cleavage, suggests that upon binding, this protein induces a structural deformation (i.e., bending and/or looping) of its target DNA.

**Deletion analysis of the *hly* regulatory region.** The above finding that the H-NS binding sites are separated by an IS2 element suggests that this random insertion of the transposable element has been positively selected because it can contribute to a better regulation of the *hly* operon. To test the validity of this hypothesis as well as the relevance of the *hlyR* sequence in the thermoregulation of the *hly* operon, we generated a series of deletions that would either eliminate the *hlyR* sequence or fuse it directly to the promoter region, thereby removing the IS2 element. Since the *hlyR* sequence contains the *ops* element, as mentioned in the introduction, two different deletions of the *hlyR* sequence were created, one (R1) lacking H-NS binding site II but still containing the *ops* element and another (R2) that completely removes the *hlyR* sequence (Fig. 1). These deletions were generated by PCR (see Materials and Methods

for details), and the resulting *Eco*RI-*Bam*HI fragments containing the deletions were cloned in the low-copy-number plasmid pLG338-30, giving rise to pLGR1, pLGR2, and pLGΔIS. These plasmids were then used to reconstruct the *hly* operon (plasmids pLGHlyR0, pLGHlyR1, pLGHlyR2, and pLGHlyΔIS) (see Materials and Methods for details). In vivo expression of the *hly* operon was then tested, and the levels of the *hly* transcript in *E. coli* cells harboring plasmids pLGHlyR0, pLGHlyR1, pLGHlyR2, and pLGHlyΔIS were determined.

Deletion of the H-NS binding site I (deletion R1) causes a partial derepression of the transcription at low temperature (Fig. 8). On the other hand, transcription is strongly reduced at 37°C when the entire *hlyR* sequence, including the *ops* element, is deleted (R2). Deletion of the IS2 element moderately reduces transcription at both temperatures.

## DISCUSSION

In this work we have extended previous observations concerning the interaction between Hha and H-NS and the role of the complex formed by these proteins in the modulation of *hly* operon expression (35). A key aspect that has been examined here concerns the role played by each protein in the generation of a nucleoprotein complex with *hly* regulatory sequences capable of modulating the expression of the operon in a temperature-dependent manner. The competition gel retardation experiments described in this work provide additional information about the interaction of both Hha and H-NS with the upstream region of the *hly* operon. Hha was found to bind to this regulatory region at a high protein/DNA ratio only, without displaying a preference for any of the fragments covering more than 4 kbp upstream of the transcription start site(s) of the *hly* operon. In contrast, H-NS was found to bind preferentially to two of the five fragments used in the assay at a protein/DNA ratio significantly lower than that required for the nonspecific DNA binding of Hha. Further characterization

```
EcoRI
|
GAATTCCAAGCGAAGTCCAT.............GTGGTCTCTGGTTTGATTTATTTGTTATTCAAGGGGGCTGTTTGGAGA
CTTAAGGTTCGCTTCAGGTA.............CACCAGAGACCAAACTAAATAAACAATAAGTTCCCCCGACAAACCTCT
1                20         181

TCGGAAAATTCTGTACGTTAAGTGTATTATTTAACCAGTTTCGATGCGTAACAGATTGATTTTGCTCAGCGGTT
AGCCTTTTAAGACATGCAATTCACATAATAAATTGGTCAAAGCTACGCATTGTCTAACTAAAACGAGTCGCCAA

ATCGCTTTTAAGTTGTTGCTCTTGGCTATCGCGTTTAGGTTATCCGATTAAAGTCAAA............TCCCGGTTTA
TAGCGAAAATTCAACAACGAGAACCGATAGCGCAAATCCAATAGGCTAATTTCAGTTT............AGGGCCAAAT
                                                         360         481

CGGGTAGTTTCCGGAAG[GGCGGTAG]CATGCTGATTCAAACTGCAAGATGA......CGTGGTGGCATCATGCTTTATG
GCCCATCAAAGGCCTTC[CCGCCATC]GTACGACTAAGTTTGACGTTCTACT......GCACCACCGTAGTACGAAATAC
                     ops                           540  661
                                                      HindIII
                                                       |
AGATAAACAATCCTGGTAGACTGGCCCCCTGAATCTCC.........GGGGATGGGGAGTAAGCTTAGTCAGCCGTTGT
TCTATTTGTTAGGACCATCTGACCGGGGGACTTAGAGG.........CCCCTACCCCTCATTCGAATCAGTCGGCAACA
                                    720  1141

CTCCGGGTGTCGCGTGCGCAGTTGCACG.........TTGTAATGGGTTAAGTGATAACAGATGTCTGGAAATATAGGG
GAGGCCCACAGCGCACGCGTCAACGTGC.........AACATTACCCAATTCACTATTGTCTACAGACCTTTATATCCC
                        1200  1981

GCAAATCCACCTGGTCAT......ATAGGCGTTACCAAAATTTGTTTGGAACTGAACAAGAAAATTGTAT[TTGTGT]AA
CGTTTAGGTGGACCAGTA......TATCCGCAATGGTTTTAAACAAACCTTGACTTGTTCTTTTAACATAAACACATT
              2040  2161                                              [-35]
CTATAATCTTAATG[TAAAAT]AAAAGACAC CAGTTCTGTAGAATATGCTTATTGAAGAGAGTGTAATAATAATTT
GATATTAGAATTAC ATTTTA TTTTCTGTGGTCAAGACATCTTATACGAATAACTTCTCTCACATTATTATTAAA
             [-10]        1

TATATAGATGT[TGTACA]AGACAGGAATGAGTAAT[TATTTA]TGCTT[GA]TGTTTTTTGACTCTTGCTTTTTATAGT
ATATATCTACA ACATGT TCTGTCCTTACTCATTA ATAAAT ACGAACTACAAAAAACTGAGAACGAAAAATATCA
         [-35]              [-10]       2

TATTATTTTTAAGTTAGTCAGCGCAATAAAAAC[TTGCTT]TTAATATTAATGCGAGT[TATGAC]ATTAAACGGAAG
ATAATAAAAATTCAATCAGTCGCGTTATTTTTGAACGAAAATTATAATTACGCTCAATACTGTAATTTGCCTTC
                                [-35]                    [-10]        3

AAACATAAAGGCATATTTTTGCCACAATATTTAATCATATAATTTAAGTTGTAGTGAGTTTATTATGAATATAA
TTTGTATTTCCGTATAAAAACGGTGTTATAAATTAGTATATTAAATTCAACATCACTCAAATAATACTTATATT
                                                                BamHI
                                                                 |
ACAAACCATT..........GCGAAAAAATTCCCTGATGAACTATTCAGAGCCATCAGGGTGGATCCCAAAACTCATGT
TGTTTGGTAA..........CGCTTTTTTTAAGGACTACTTGATAAGTCTCGGTAGTCCCACCTAGGGTTTTGAGTACA
     2520     2821                                                        2880
```
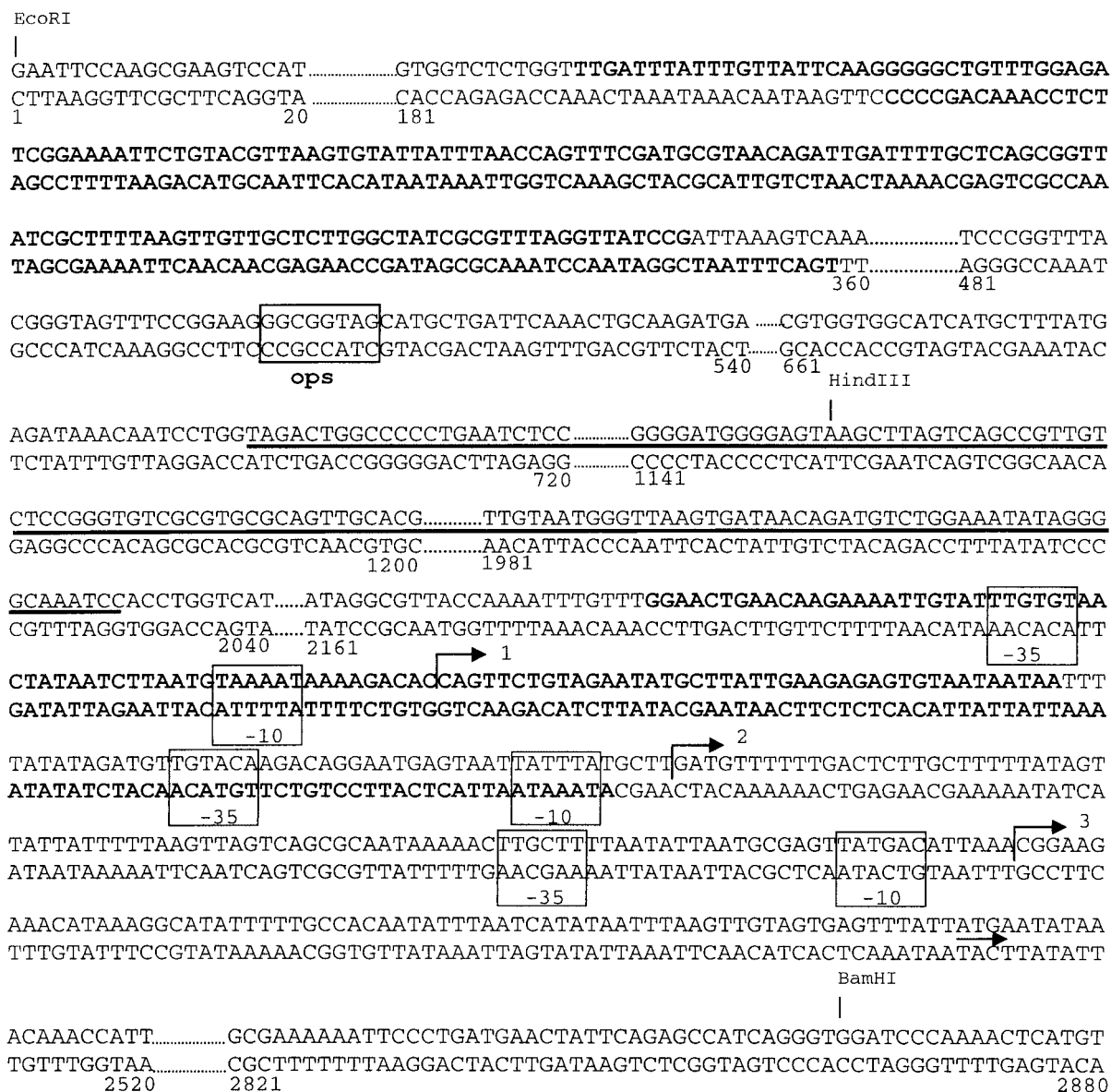
FIG. 6. Location of the H-NS binding sites within the nucleotide sequence of *hly* regulatory region. The sequences protected by H-NS protein are in bold letters. The IS*2* sequence is underlined and the *ops* element is indicated by the corresponding box. The −35 and −10 elements of three promoters identified for the *hly* operon are indicated by the corresponding boxes. Bent arrows and the short arrow indicate the transcription start sites and the first codon of *hlyC*, respectively. Restriction sites for *Eco*RI, *Hin*dIII, and *Bam*HI are also indicated.

by DNase I footprinting of the H-NS binding preference allowed us to identify two fairly extended binding sites (sites I and II), the first within the *hlyR* sequence (nucleotides 190 to 350) and the second (nucleotides 2180 to 2330) partially overlapping the promoter region of the *hly* operon of plasmid pHly152 (Fig. 6). The relevance of site I for thermoregulation is evidenced by the results of the deletion analysis of the *hly* operon that we have performed in the present study.

The results of two different approaches (computational prediction and AFM analysis) indicated the existence of an intrinsic DNA curvature in the region separating the two H-NS sites. The occurrence of an intrinsic curvature separating two H-NS binding sites has been previously reported in the upstream region of genes

subjected to temperature-independent (*hns* and *cspA*) and temperature-dependent (*virF*) transcriptional repression by H-NS (3, 10, 11). A significant difference between the thermoregulated *virF* gene (11) and the *hly* operon is the presence in the latter of an IS*2* sequence separating the two H-NS binding sites, thereby causing a more extensive separation between them. In any event, the role of the IS*2* element in expression of the *hly* operon could not be clarified by removing it from its insertion site. As no *hly* promoter function has hitherto been assigned to this element, it is apparent that the moderate effect of IS*2* deletion on *hly* transcription is most likely due to the fact that when the two H-NS binding sites are separated, topological modifications in the region are introduced.
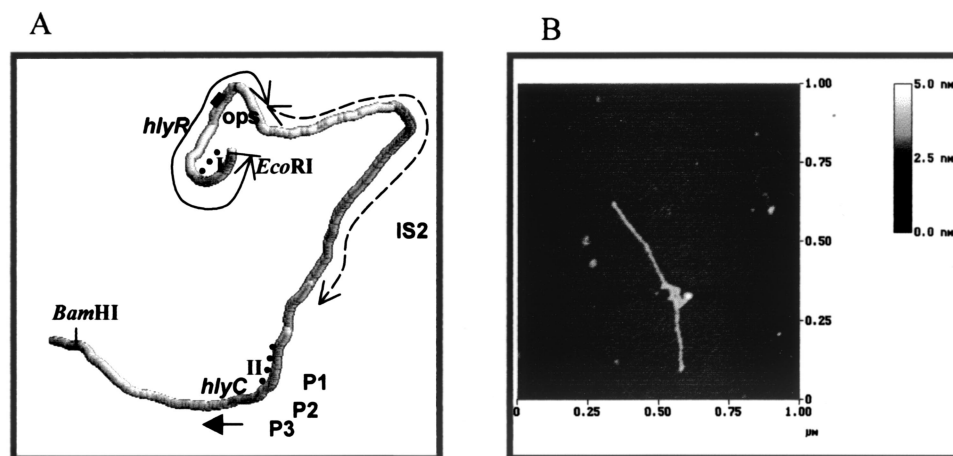
A



B



FIG. 7. (A) Computer-generated prediction of the intrinsic curvature of the 3.1-kbp fragment covering the *hly* regulatory region and part of the structural genes. Dotted lines indicate the H-NS binding sites I and II. (B) AFM image in dry air of the 2.8-kbp *Eco*RI-*Bam*HI fragment in HEPES-MgCl$_2$ buffer adsorbed on bare mica (see Materials and Methods for details). The molecule shown is representative of many others observed.

It is apparent that temperature influences the affinity of H-NS for its target sequences within the regulatory region of the *hly* operon. In fact, the higher affinity of H-NS for the 2.8-kb *Eco*RI-*Bam*HI fragment at low temperature correlates well with the more efficient transcriptional repression caused by H-NS at low temperature and with the hemolytic phenotype of the *hns* mutants. Taken together, these results indicate that H-NS is the protein that confers specificity to the regulatory loop by eliciting the temperature-dependent formation of a nucleoprotein complex comprising the regulatory sequences of *hly*.

With respect to Hha, different lines of experimental evidence suggest that it probably acts as a protein binder rather than as a DNA binder: (i) it shows strong affinity in vitro for H-NS (35) and (ii) a mutational analysis has shown that almost all of the protein present is required to bind H-NS (34). There is a certain degree of homology between the amino acid sequence of the described proteins from the Hha family and the oligomerization domain of the H-NS family of proteins (34). Considering the phenotype of *hha* mutants (partial derepression of temperature- and osmolarity-mediated hemolysin expression) and the role of this protein as an H-NS binder, it appears reasonable that the role of Hha in modulation of *hly* expression is accomplished by binding to H-NS to generate a hetero-oligomeric complex that represses *hly* transcription more efficiently than H-NS oligomers alone.

The mechanism underlying temperature-mediated repression of *hly* operon can be modeled from the data reported in this paper. At low temperature, the increased affinity of H-NS for the two identified binding sites generates nucleoprotein complexes that include Hha as well. Because of the increased flexibility of DNA at low temperature (the increased flexibility being a consequence of a higher degree of DNA supercoiling), H-NS molecules located at both sites may contact, thereby allowing the generation of a larger nucleoprotein complex which includes H-NS and Hha. That complex would occlude both the *ops* sequence and the promoter region, thus repressing transcription and abolishing the antitermination effect of *ops*. An increase in the growth temperature may have two different effects: (i) reduction of the affinity of H-NS for its target sequences and (ii) reduction of the flexibility of the DNA. As a possible consequence, H-NS molecules colliding with their target sequences are not able to generate nucleoprotein complexes large enough to extend between them and the decrease in DNA flexibility does not allow it to stabilize a nucleoprotein complex which includes both sites. Under the latter circumstances, transcriptional repression is at least partially alleviated. A delicate balance between the moderate affinity of H-NS for its target sites and the effect of temperature on DNA structure and flexibility probably underlies thermoregulation of the *hly* operon. It is relevant to mention here that growth at 37°C does not result in complete derepression of *hly* expression. H-NS still partially represses transcription, as demonstrated by the significant increase in hemolysin expression that is evidenced in *hns* mutants growing at 37°C (35). This means that H-NS- and Hha-mediated repression of the *hly* operon is not an on-off process but, rather, a gradual one, in which different environmental factors that cause variations in the physicochemical parameters of DNA or sequestering or degradation of H-NS modify hemolysin expression.

The model presented here is strongly reminiscent of that previously reported for the thermoregulation of the *virF* operon (11) and hence supports a general model for H-NS-mediated thermoregulation of virulence gene expression. In
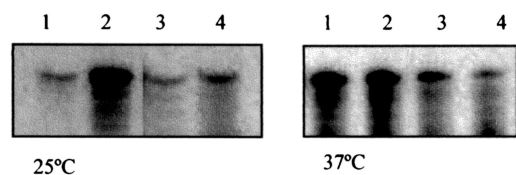


FIG. 8. Effect of different deletions on transcription of the *hly* operon. RNase protection assay of *hlyC* transcripts from mid-exponential-phase cultures of *E. coli* 5K, grown at 25 or 37°C and carrying pLGHlyR0 (lanes 1), pLGHlyR1 (lanes 2), pLGHlyR2 (lanes 3), or pLGHlyΔIS (lanes 4). Representative results are shown.

addition, it is completely compatible with the previous reported models for H-NS-mediated repression of transcription. H-NS molecules generating a nucleoprotein complex that represses transcription fit with the promoter-occlusion model, and the interaction of H-NS molecules with DNA to modify the level of supercoiling and hence DNA flexibility fits with the model supporting a role for H-NS on DNA topology (11, 39, 40). This means that H-NS could play a dual role, both as a protein specifically modifying DNA structure and as a repressor of transcription.

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