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Regulation of the *Escherichia coli* K5 Capsule Gene Cluster: Evidence for the Roles of H-NS, BipA, and Integration Host Factor in Regulation of Group 2 Capsule Gene Clusters in Pathogenic *E. coli*

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The expression of *Escherichia coli* group 2 capsules (K antigens) is temperature dependent, with capsules only being expressed at temperatures above 20°C. Thermoregulation is at the level of transcription, with no detectable transcription at 20°C. Using the *E. coli* K5 capsule gene cluster as a model system, we have shown that the nucleoid-associated protein H-NS plays a dual role in regulating transcription of group 2 capsule gene clusters at 37 and 20°C. At 37°C H-NS is required for maximal transcription of group 2 capsule gene clusters, whereas at 20°C H-NS functions to repress transcription. The BipA protein, previously identified as a tyrosine-phosphorylated GTPase and essential for virulence in enteropathogenic *E. coli*, was shown to play a similar role to H-NS in regulating transcription at 37 and 20°C. The binding of integration host factor (IHF) to the region 1 promoter was necessary to potentiate transcription at 37°C and IHF binding demonstrated by bandshift assays. The IHF binding site was 3′ to the site of transcription initiation, suggesting that sequences in the 5′ end of the first gene (*kpsF*) in region 1 may play a role in regulating transcription from this promoter at 37°C. Two additional *cis*-acting sequences, conserved in both the region 1 and 3 promoters, were identified, suggesting a role for these sequences in the coordinate regulation of transcription from these promoters. These results indicate that a complex regulatory network involving a number of global regulators exists for the control of expression of group 2 capsules in *E. coli*.

Escherichia coli produces more than 80 chemically and serologically distinct capsules, called K antigens (18). These capsules have been separated into four groups on the basis of chemical composition, molecular weight, intergenic relationships, and regulation of expression (6, 23, 34). The majority of extraintestinal isolates of *E. coli* associated with invasive disease express group 2 capsules, with particular capsules being associated with certain diseases (17). Typical of many virulence factors, expression of group 2 capsules in *E. coli* is regulated by temperature, with group 2 capsules only being expressed at temperatures above 20°C (18, 34). Environmental regulation of virulence gene expression is well documented in a broad range of pathogenic bacteria (19), and it is likely that this offers a mechanism by which pathogenic bacteria may adapt to particular niches encountered within the host. The regulation of virulence gene expression by temperature provides a means by which bacteria may selectively express virulence determinants upon entry into the host.

Group 2 capsule gene clusters have a common organization consisting of three regions (25, 34). A central, capsule-specific region, region 2, encoding the enzymes for polysaccharide biosynthesis (24), is flanked by regions 1 and 3, which are common to all of the group 2 capsule gene clusters. Regions 1 and 3 encode the eight Kps proteins, which constitute the common export pathway for the transport of group 2 polysaccharides out of the cell (5, 22, 25, 26, 34). Region 1 consists of six genes, *kpsFEDUCS*, organized in a single transcriptional unit with a σ^{70} promoter which is located 225 bp 5′ of *kpsF* (4, 22, 29). Two integration host factor (IHF) binding site consensus sequences were identified 110 bp 5′ and 130 bp 3′ of the transcription start site (29). Transcription from the promoter 5′ of *kpsF* generates an 8.0-kb polycistronic transcript that is then processed to generate a separate, 1.3-kb *kpsS* transcript which may enable the differential expression of the KpsS protein (29).

Region 3 contains two genes, *kpsM* and *kpsT*, which are organized as a single transcriptional unit with a σ^{70} promoter 741 bp 5′ of the start of *kpsM* (30). An *ops* sequence, which is conserved in RfaH-regulated genes, is located 30 bp 5′ to the initiation codon of *kpsM*, and mutations in *rfaH* or deletion of the *ops* sequence results in a lack of capsule expression (30). By quantitative reverse transcriptase-PCR, it has been shown that RfaH acts to allow readthrough transcription originating from the region 3 promoter to proceed into region 2 and that readthrough transcription is essential for expression of the region 2 genes and group 2 capsule production (30). As such, the transcriptional organization of group 2 capsule gene clusters can be regarded as two large convergent transcripts, one of which originates from the region 1 promoter and covers region 1, and the other originating from the region 3 promoter and spanning regions 2 and 3.

Although previously it had been shown that at 20°C there is no transcription from the region 1 promoter (5, 29), little was known about the regulation of transcription of group 2 capsule gene clusters. In this study we demonstrate that the global regulator H-NS and the tyrosine-phosphorylated GTPase BipA are essential for maximal transcription from the region 1 and 3 promoters at 37°C. Paradoxically, both proteins also function in the temperature regulation of these promoters, repressing transcription at 20°C. In addition, we demonstrate the binding of IHF to the region 1 promoter and identify the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
MC4100*hns*	*hns*::*kan*	Jay Hinton
MS101	$K5^+$	30
MS150	MS101 Δ(*arg-lacZ*)	29
MS151	MS150 *himA*::*cat*	This study
MS152	MS150 *hns*::*kan*	This study
MS153	MS150 *bipA*::Tn*5*	This study
HN880	IHF-overproducing strain	21
Plasmids		
pDSHcH	1.1-kb *Hin*cII-*Hin*dIII fragment containing region 1 promoter cloned into promoter probe plasmid pCB192	29
pCBIHF-1	pDSHcH with a 13-bp deletion removing the IHF1 site	This study
pCBIHF-2	pDSHcH with a 12-bp deletion removing the IHF2 site	This study
pSR4A	pDSHcH with a 14-bp deletion removing the conA site	This study
pSR4B	pDSHcH with a 17-bp deletion removing the conB site	This study
pSR4AB	pDSHcH with a 46-bp deletion removing both the conA and conB sites	This study

IHF binding site and other *cis*-acting regulatory sequences important for transcription at 37°C.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains and plasmids used in this study are shown in Table 1. Bacteria were grown in Luria (L) broth supplemented with ampicillin (100 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), kanamycin (50 μg ml^{-1}), or streptomycin (100 μg ml^{-1}) where appropriate at either 37 or 20°C. Tn*5* mutagenesis was performed as described before (7). The site of Tn*5* insertion was determined by constructing a plasmid library of the transposon insertion mutant, followed by screening the library for clones resistant to kanamycin. The nucleotide sequence flanking the site of insertion was then determined using primers specific to the ends of Tn*5*. Where appropriate, mutations were introduced into strains by P1*vir* transduction (30), and transductants were selected on L-agar supplemented with kanamycin (50 μg ml^{-1}).

DNA procedures. Plasmid DNA was prepared by alkaline lysis (27). Deletion of the *conA* and *conB* sites in the region 1 promoter was generated by inverse PCR mutagenesis (IPCRM) as described before (14) with the following primers; SR1, 5′-AAATCCTAGGTACCTTGTTCATAATG-3′; SR2, 5′-AAATGGTACCTACTTGACTATTAATAC-3′; SR3, 5′-CAAGTAGGAAACATTTTAACAAATGATA-3′; SR4, 5′-CCAAAAACAATTTATCAATTGATTATTTTC-3′; SR6, 5′-AAATGTTTCCTACTTGACTATTAATAC-3′; and SR8, 5′-CCTAAATTCCTTGTTCATAATGTAGGA-3′. Deletion of the putative IHF binding sites IHF1 and IHF2 was achieved using the primers IHF1D1 (5′-TAGCATAAATAAATTATAGTGGTT-3′) and IHF1D2 (5′-TAAACAAAATTTTTAATGAATATAAAACCAT-3′) for site IHF1 and IHF2D1 (5′-AAATTGTTTTTGGTATTAATAGTCAAG-3′) and IHF2D2 (5′-TTTTCTTGTAAAAAAAGAACGTATGA-3′) for site IHF2.

RNA procedures. RNA was prepared by hot acid-phenol extraction (1). To quantitate the specific mRNAs, 50 μg of total RNA was applied to nylon membranes (Hybond N) using a Bio-Rad Biodot-SF apparatus and hybridized with DNA probes labeled with [α-^{32}P]dCTP and random hexanucleotides. The signals due to bound probe were detected and quantified with a FujiX BAS2000 Phosphoimager with Aida V2.0 software. Hybridization with a radiolabeled probe containing the 5s RNA gene was used to confirm the total loading of RNA onto the filter.

Enzyme assays. Bacterial strains were grown to mid-logarithmic phase (optical density at 600 nm of 0.5) at either 37 or 20°C, and where appropriate, β-galactosidase activity was assayed as described before (20).

Gel retardation experiments. Two 200-bp [α-^{32}P]dCTP-radiolabeled PCR fragments containing the putative IHF binding sites were amplified using either primers IHF1F (5′-CAACCTGTTTATTATGCC-3′) and IHF1R (5′-CACACTCCTACATTATGA-3′) for the IHF1 site or primers IHF2F (5′-GCACCTCCATGAGACATT) and IHF2R (5′-CCAGGTAAATGTCTTTCAGGAC-3′) for the IHF2 site. The particular radiolabeled PCR product was incubated with appropriate cell lysates or purified IHF (20 mM), and the binding of IHF to the PCR fragment was monitored by gel retardation assays as described before (28).

Measurement of extracellular K5 polysaccharide. The amount of extracellular K5 polysaccharide produced by MS150 and its derivatives was quantified using the carbazole assay (3) with purified K5 polysaccharide as a standard. Since K5 is the only extracellular uronic acid-containing polysaccharide produced by MS150, this assay is an accurate measure of the relative amounts of K5 polysaccharide.

RESULTS AND DISCUSSION

Analysis of the role of IHF on transcription from the region 1 promoter. Two putative IHF binding sites, termed IHF1 and IHF2, had previously been identified in the region 1 promoter (Fig. 1), and mutations in the *himA* gene were shown to reduce expression of the KpsE protein (29). To demonstrate the effect of IHF on transcription from the region 1 promoter, plasmid pDSHcH (Table 1) was introduced into strains MS150 and MS151, and the level of β-galactosidase was assayed following growth at 37°C. Strain MS150(pDSHcH) expressed 1,398 ± 97 (mean ± standard error of the mean [SEM]) U, whereas strain MS151(pDSHcH) expressed 154 ± 39 U, a sevenfold reduction in the level of β-galactosidase. To demonstrate the functional importance of the IHF1 and IHF2 binding sites, IPCRM was undertaken to generate plasmids pCBIHF-1 and pCBIHF-2 (Table 1), in which IHF1 or IHF2, respectively, was deleted (Fig. 1). These plasmids were introduced into MS150, and the level of β-galactosidase was assayed. Deletion of either IHF1 or IHF2 reduced β-galactosidase activity by twofold; strain MS150(pDSHcH) expressed 933 ± 43 U, compared with strains MS150(pCBIHF-1) (519 ± 45 U) and MS150 (pCBIHF-2) (478 ± 33 U).

The interaction of IHF with the region 1 promoter was shown by gel shift assays. A radiolabeled PCR fragment spanning IHF1 was amplified from pDSHcH using primers IHF1F and IHF1R (Fig. 1) and incubated with extracts from strains HN880, MS150, and MS151 and with purified IHF (Fig. 2). In the case of both strains HN880 and MS150 and with purified IHF, a reproducible band shift could be detected which was absent when the fragment was incubated with extracts from strain MS151 (Fig. 2). This demonstrates that IHF is binding to the region 1 promoter. To localize the site of interaction between IHF and the region 1 promoter, the same primers were used to amplify the corresponding fragment from plasmid pCBIHF1, in which the IHF1 site had been deleted by IPCRM (Table 1). This PCR fragment lacking IHF1 was then used in gel retardation assays. Deletion of IHF1 abolished the band shift (Fig. 2), confirming the binding of IHF at this site in the region 1 promoter. Primers IHF2F and IHF2R were then used to amplify a radiolabeled PCR fragment containing the IHF2 site (Fig. 1). However, no IHF-dependent band shifts were detectable with this fragment in gel retardation experiments (data not shown).

The identification of an IHF binding site at +130 is a sur-

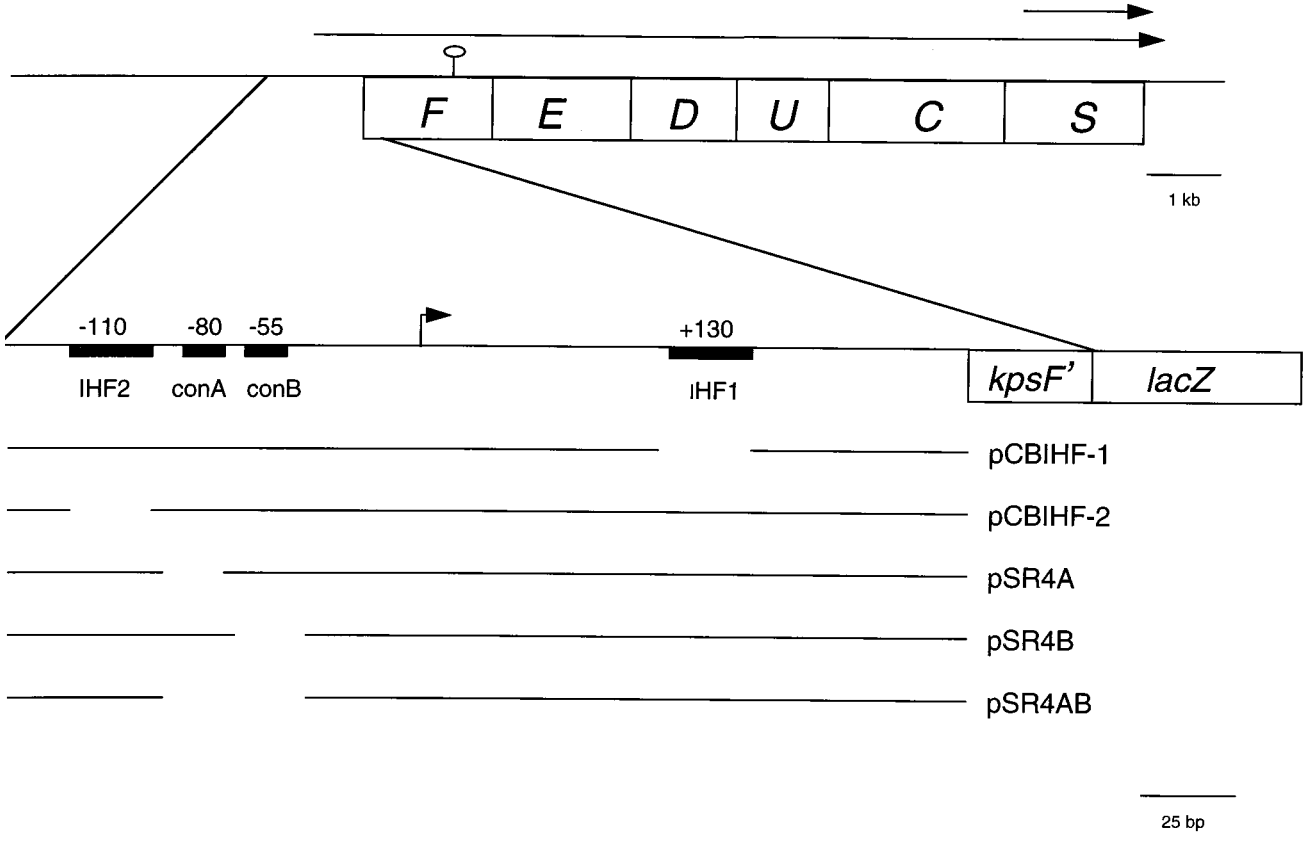

FIG. 1. Analysis of the region 1 promoter. The top line depicts region 1, with the larger arrow denoting the 8.0-kb polycistronic transcript and the smaller arrow denoting the processed *kpsS*-specific transcript. The stem-loop structure shows the intragenic Rho-dependent terminator within the *kpsF* gene. At the bottom, the promoter region is enlarged. The IHF1, IHF2, conA, and conB sites are boxed, and the numbers state the distance in base pairs from the center of the site to the transcriptional start site, depicted by an arrow.

prising result, since IHF binding sites are usually 5′ to the site of transcription initiation (16). The only other reported example of an IHF binding site 3′ to the site of transcription initiation is in the Pc promoter of bacteriophage Mu (32). Generally IHF acts in concert with other transcriptional factors (16) and is believed to bend the DNA within the promoter region, to facilitate the appropriate DNA-protein interactions necessary for transcriptional activation (9, 15, 16). In the case of the region 1 promoter, introducing a bend in the DNA centered on the first residue of the IHF1 site would suggest that sequences 3′ to IHF1, possibly in the 5′ end of the *kpsF* gene, may be important in the transcriptional activation of the region 1 promoter at 37°C. The observation that a *himA* mutation had a more dramatic effect on region 1 transcription than deletion of the IHF1 site suggests that IHF may also play an indirect role in the activation of region 1 transcription, perhaps by controlling the expression of an additional *trans*-acting regulator.

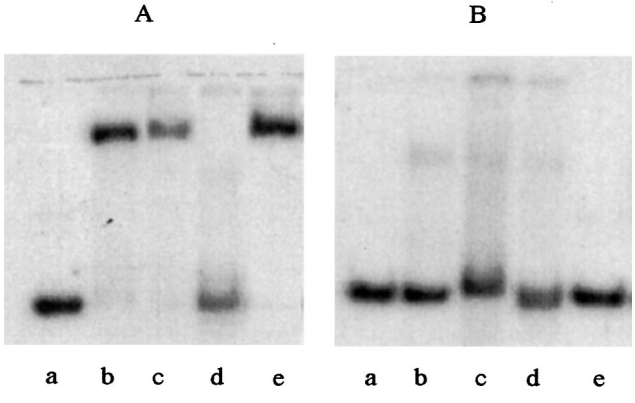

FIG. 2. Gel retardation experiments. Cell lysates from strains HN880 (lane b), MS150 (lane c), and MS151 (lane d), along with purified IHF at a final concentration of 20 nM (lane e), were incubated with 0.5 pmol of [^{32}P]dCTP-labeled PCR product prepared from either (A) pDSHcH (IHF1$^+$) or (B) pCBIHF1 (IHF1$^-$). Following incubation, the samples together with an aliquot of the PCR product (lane a) were analyzed by electrophoresis followed by autoradiography.

Identification of *cis*-acting elements in the region 1 promoter. Comparison of the region 1 and 3 promoters identified two conserved sequences, termed conA and conB, which are separated by 17 bp and are located 5′ to the −35 region in both promoters (29). It has been suggested that these sequences could represent *cis*-acting regulatory sequences that are important in coordinating transcription from the region 1 and 3 promoters (25, 29). To elucidate the possible role of these sequences, IPCRM was used to generate plasmids pSR4A, pSR4B, and pSR4AB, from which conA, conB, and both conA and conB have been deleted, respectively (Fig. 1, Table 1). These plasmids were introduced into strain MS150, and the level of β-galactosidase at 37 and 20°C was assayed. Deletion of either or both conA and conB reduced β-galactosidase activity by greater than twofold but had no effect on stimulating transcription at 20°C (Table 2). One interpretation of these

TABLE 2. Effect of deleting conA and conB on transcription from the region 1 promoter

Strain	β-Galactosidase activity[a]	
	37°C	20°C
MS150(pDSHcH)	1,398 ± 97	222 ± 15.28
MS150(pSR4A)	341 ± 83	138 ± 18
MS150(pSR4B)	430 ± 75	95 ± 7
MS150(pSR4AB)	526 ± 78	162 ± 13

[a] Activity is expressed in Miller units ± the SEM.

TABLE 4. Effect of *hns*, *bipA*, and *himA* mutations on extracellular K5 polysaccharide production at 37 and 20°C

Strain	Mean extracellular K5 polysaccharide (μg ml^{-1}) ± SEM	
	37°C	20°C
MS150	20.19 ± 0.42	2.21 ± 0.08
MS151 (*himA*::*cat*)	4.24 ± 0.30	2.15 ± 0.04
MS152 (*hns*::*kan*)	11.18 ± 0.74	11.95 ± 0.21
MS153 (*bipA*::Tn*5*)	9.14 ± 0.78	12.78 ± 0.30

data is that the deletions are having a generic effect on disrupting promoter function. However, this is unlikely to be the case for two reasons. First, it has been shown that sequences 5′ to −50 are not important for the binding of RNA polymerase and the initiation of transcription from constitutive σ^{70} promoters (8). Second, in regulated σ^{70} promoters, such 5′ sequences define *cis*-acting sites for the binding of regulatory proteins (8, 10). As such, the most likely interpretation is that conA and conB define a *cis*-acting region necessary for maximum transcription from the region 1 promoter. The proximity of IHF2 to conA and conB (Fig. 1), deletion of which also reduces transcription by twofold, would be in keeping with this notion. The conservation of conA and conB in the region 3 promoter could represent a mechanism to coordinate transcription from the region 1 and 3 promoters at 37°C.

Role of H-NS and BipA in transcription from the region 1 and 3 promoters. The global regulator H-NS has been shown to play a key role in the temperature regulation of a number of virulence genes (2). To investigate the role of H-NS in the temperature regulation of the region 1 promoter, plasmid pDSHcH was introduced into strain MS152, which contains the *hns*::*kan* null mutation from strain MC4100 (Table 1), and the level of β-galactosidase activity was assayed following growth at 37°C. Strain MS150(pDSHcH) expressed 1,030 ± 21 U, whereas strain MS152(pDSHcH) expressed 404 ± 36 U. These results were confirmed by RNA dot blot analysis with a *kpsE*-specific probe to RNA extracted from MS152 grown at 37 and 20°C (Table 3). The *hns* mutation resulted in comparable levels of transcription at both 20 and 37°C (Table 3), indicating that H-NS was required for maximal transcription at 37°C as well as acting to repress transcription at 20°C. To determine the effect of the *hns* mutation on capsule production, the levels of extracellular K5 polysaccharide produced by MS152 were determined. The *hns* mutation reduced the extracellular K5 polysaccharide level by 50% at 37°C but increased K5 polysaccharide production at 20°C, with comparable levels of K5 polysaccharide being made at both temperatures (Table 4).

TABLE 3. Quantitative RNA dot blot analysis of region 1 and 3 transcription

Strain	Band intensity[a] with probe:			
	kpsE		*kpsT*	
	37°C	20°C	37°C	20°C
MS150	100 ± 5	19 ± 2	100 ± 5	17 ± 2
MS152 (*hns*::*kan*)	52 ± 10	52 ± 4	45 ± 10	38 ± 4
MS153 (*bipA*::Tn*5*)	20 ± 8	49 ± 6	26 ± 6	45 ± 6

[a] The signals were quantified as described in Materials and Methods and are expressed as a percentage of the most intense signal (MS150 at 37°C) for each probe used. The experiment was repeated three times, and results are expressed ± the SEM. The region 1 (*kpsE*) and region 3 (*kpsT*)-specific probes were used to hybridize to RNA extracted from cells grown at either 37 or 20°C.

To identify other regulatory genes, plasmid pDSHcH was introduced into a pool of Tn*5* mutants of MS150, and colonies expressing reduced β-galactosidase activity were identified on medium supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Two colonies were identified, and in both cases the Tn*5* had inserted at different sites into the *E. coli bipA* gene. One of these strains, termed MS153 (Table 1), was used for further study, and the level of β-galactosidase was assayed. The *bipA* mutation reduced β-galactosidase activity by fivefold in cells grown at 37°C, 1,030 ± 21 U for strain MS150(pDSHcH) versus 182 ± 43 U for strain MS153 (pDSHcH). The effects of the *bipA* mutation were confirmed by RNA dot blot analysis with a *kpsE*-specific probe to RNA extracted from MS150 and MS153 grown at 37 and 20°C. At 37°C the *bipA* mutation caused a threefold reduction in transcription from the region 1 promoter (Table 3), while at 20°C the *bipA* mutation increased transcription to levels comparable to those seen in an *hns* mutant (Table 3). The effects of the *bipA* mutation on region 1 transcription were mirrored in the levels of extracellular K5 polysaccharide produced at 37 and 20°C (Table 4).

Quantitative RNA dot blots with the *kpsT* gene as a radiolabeled probe to RNA extracted from MS150, MS152, and MS153 grown at 37 and 20°C confirmed that H-NS and BipA regulated transcription from the region 3 promoter. At 37°C the *hns* mutation reduced region 3 transcription twofold, while the *bipA* mutation had a greater effect, reducing transcription more than threefold (Table 3). At 20°C both the *hns* and *bipA* mutations increased transcription twofold from the region 3 promoter (Table 3).

H-NS has been implicated in the temperature regulation of a number of genes, with H-NS repressing gene expression at low temperature (2, 11, 31, 33). Generally, *hns* mutations increase transcription at both the permissive and nonpermissive temperatures to comparable levels. In the case of the region 1 and 3 promoters, the situation is more complex, since the *hns* mutation has a more modest effect on increasing transcription at 20°C and does not completely abolish temperature regulation (Table 3). In addition to its effect on transcription at 20°C, the *hns* mutation reduced transcription from both the region 1 and 3 promoters at 37°C by twofold, as assayed by reporter gene activity and quantitative RNA dot blot analysis. Taken together, these data provide compelling evidence that H-NS is required for maximal transcription from both the region 1 and 3 promoters at 37°C. The effect of the *hns* mutation was mirrored in reduced levels of extracellular K5 polysaccharide at 37°C (Table 4). The observation that H-NS has a dual role, being involved in the activation of group 2 capsule gene expression at 37°C while also playing a role in repression at 20°C, is unique for H-NS-mediated thermoregulation. At this stage,

the mechanism by which H-NS functions in this dual role is unclear.

The isolation of mutants that have transposon insertions in the *bipA* gene and are altered in transcription of both the region 1 and 3 promoters at 37 and 20°C is intriguing. The BipA protein was identified as a protein that was phosphorylated on a tyrosine residue in enteropathogenic *E. coli* (EPEC) strains (12, 13). In addition, EPEC *bipA* mutants do not trigger cytoskeletal rearrangements in host cells, are hypersensitive to the host defense protein BPI, and demonstrate increased mobility and flagellum expression (12). The observation that the *E. coli* BipA protein has GTPase activity and is homologous to both elongation factor G and the TetO resistance protein, both of which interact with the ribosome, leads to the suggestion that the BipA protein may represent a new class of regulators which function via interactions with the ribosome (12). The effect of the *bipA* mutation on transcription from the region 1 and 3 promoters is unlikely to be via a direct interaction between the BipA protein and the respective promoter, and the most likely explanation is that BipA regulates the activity of proteins that are required for the regulation of transcription of the region 1 and 3 promoters.

In summary, we can begin to develop a model to explain the regulation of group 2 capsule gene expression in pathogenic *E. coli*. At 37°C the region 1 and 3 promoters are active, and both H-NS and BipA are required for maximal transcription. The identification of *cis*-acting sequences that are conserved within the region 1 and 3 promoters would suggest that additional regulatory proteins interact to stimulate transcription at 37°C. In the case of the region 1 promoter, there is an additional requirement for IHF, which is likely to be providing an architectural role in bending the DNA to allow the assembly of the nucleoprotein complex. In the case of region 3, RfaH will be interacting with the RNA polymerase complex to permit transcription elongation to proceed from region 3 into region 2. Paradoxically, at 20°C, both H-NS and BipA also play roles in repressing transcription, suggesting key roles for these proteins in the regulation of group 2 capsule expression. Studies are ongoing to further elucidate this complex regulatory circuit and establish roles for additional regulatory proteins.

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