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Integration Host Factor Positively Regulates Virulence Gene Expression in *Vibrio cholerae*[▽]

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Virulence gene expression in *Vibrio cholerae* is dependent upon a complex transcriptional cascade that is influenced by both specific and global regulators in response to environmental stimuli. Here, we report that the global regulator integration host factor (IHF) positively affects virulence gene expression in *V. cholerae*. Inactivation of *ihfA* and *ihfB*, the genes encoding the IHF subunits, decreased the expression levels of the two main virulence factors *tcpA* and *ctx* and prevented toxin-coregulated pilus and cholera toxin production. IHF was found to directly bind to and bend the *tcpA* promoter region at an IHF consensus site centered at position −162 by using gel mobility shift assays and DNase I footprinting experiments. Deletion or mutation of the *tcpA* IHF consensus site resulted in the loss of IHF binding and additionally disrupted the binding of the repressor H-NS. DNase I footprinting revealed that H-NS protection overlaps with both the IHF and the ToxT binding sites at the *tcpA* promoter. In addition, disruption of *ihfA* in an *hns* or *toxT* mutant background had no effect on *tcpA* expression. These results suggest that IHF may function at the *tcpA* promoter to alleviate H-NS repression.

The ability of pathogenic bacteria to modulate the expression of genes in response to environmental stimuli is widespread and critical for bacterial survival within specific niches. Nucleoid-associated proteins, such as H-NS, integration host factor (IHF), Fis, and HU play important roles in this adaptive process. These proteins comprise a group of abundant DNA-binding proteins that condense the bacterial chromosome, modulating its structure and influencing many cellular processes, such as DNA replication (51), site-specific recombination (5, 16), and transcription (35). The nucleoprotein complexes formed in response to environmental cues have both negative and positive influences on transcription, and these proteins function with one another and with gene-specific regulatory factors to fine tune gene expression.

The nucleoid-associated protein H-NS is an abundant global regulator that influences various cellular processes (13, 14, 41). H-NS binds DNA with relatively low sequence specificity but instead has a preference for AT-rich segments with intrinsic planar curvature (49, 68). Studies using chromatin immunoprecipitation-on-chip technology have identified the H-NS binding regions over the *Escherichia coli* and *Salmonella enterica* serovar Typhimurium genomes (18, 32, 42, 44). Their results demonstrate that H-NS binds to regions of high AT content, including genes acquired by horizontal gene transfer. Of interest was the finding that H-NS interacts with extensive DNA stretches on pathogenicity islands and represses the transcription of the associated virulence genes. This discovery has raised speculation that H-NS may function to silence foreign DNA until dedicated transcription factors are able to relieve H-NS repression (14). Recent studies have proposed that H-NS dimers bind to nucleating high-affinity sites, corresponding with the identified 10-bp consensus motif TCGATAAATT, located within AT-rich regions, and polymerize outward along the DNA, eventually resulting in the condensation of DNA through the formation of protein bridges between H-NS binding sites (4, 29). This DNA remodeling can directly interfere with RNA polymerase action or block the binding of specific activators at a nearby promoter region.

IHF is a heterodimeric protein composed of the subunits IHFα (11.3 kDa) and IHFβ (10.6 kDa), encoded by the *ihfA* (*himA*) and *ihfB* (*hipB*) genes, respectively. The two subunits have approximately 35% identity to each other. Unlike H-NS, IHF recognizes a specific asymmetric site on DNA, the 13-bp consensus target sequence WATCAANNNNTTR, where W represents A or T and R represents A or G (10). Upon binding, IHF is known to bend DNA by as much as 180° (48, 61). In *E. coli* and *S. enterica* serovar Typhimurium, IHF is involved in controlling the transcriptions of over 100 genes of various functions (2, 33, 35). In addition, IHF has increasingly been identified as a regulator of virulence gene expression in various pathogens. IHF is required for appropriate expression of the *Brucella abortus virB* operon, encoding the type IV secretion system (52). In *Shigella flexneri*, IHF positively regulates virulence gene expression at multiple promoters, including the *virF*, *virB*, and *icsA* promoters (47). Flagellum production in enteropathogenic and enterohemorrhagic *E. coli* strains is repressed by IHF (69). IHF can act as a transcriptional regulator by different mechanisms. In several cases, IHF produces a DNA bend that enables the direct interaction of a distant, DNA-bound transcriptional regulator with RNA polymerase to enhance transcription (15). DNA bending by IHF can stimulate the recruitment of RNA polymerase to activate transcription by favoring the interaction of the α subunit C-terminal domain with upstream DNA UP elements (3). IHF binding can also serve to lower the activation energy for open complex formation (45). IHF typically acts as an architectural factor

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that leads to the correct promoter structure necessary for transcriptional regulation. An alternative mechanism of IHF activation is antirepression of H-NS-repressed promoters (63).

Virulence gene expression in the human enteric pathogen *Vibrio cholerae* is dependent upon a complex transcriptional cascade that is influenced by both specific regulators, such as ToxR/S, TcpP/H, and ToxT, and global regulators, such as cyclic AMP-cyclic AMP receptor protein and H-NS, in response to environmental stimuli (for reviews, see references 9 and 55). Production of the two main virulence factors, the *t*oxin-*c*oregulated *p*ilus (TCP) (60) and the *c*holera *t*oxin (CT) (23), is required for human colonization and causes the severe watery diarrhea that is characteristic of the disease. Both of these virulence factors are carried on genetic elements acquired by horizontal gene transfer that have a higher AT content (>60% AT rich) than the ancestral chromosome (52% AT rich). The *tcp* genes are carried on the *Vibrio p*athogenicity *i*sland (VPI), and the *ctx* genes are carried on the CT phage. The expression of the *tcp* and *ctx* genes is transcriptionally controlled in response to environmental stimuli by a regulatory cascade, termed the ToxR virulence regulon, that involves proteins encoded within both the ancestral *Vibrio* genome and the VPI. The cascade is initiated by two chromosomally encoded proteins, AphA and AphB, functioning synergistically to activate transcription of *tcpPH* (25, 56). TcpP and TcpH (6, 20) are transmembrane proteins encoded on the VPI that, along with a second pair of transmembrane proteins, ToxR and ToxS (37, 38), activate transcription at the *toxT* promoter in a cooperative manner (21, 28). ToxT directly activates the expression of many genes, including the *tcp* operon (7, 12, 22, 71), the *ctx* operon (12), and the *a*ccessory *c*olonization *f*actor genes *acfA* and *acfD* (46, 65). In addition, ToxT autoregulates its own expression through activation of the *tcpA* promoter and readthrough into *toxT* (70).

In addition to the specific regulatory proteins of the ToxR virulence regulon mentioned above, the nucleoid-associated protein H-NS has been shown to play an important role in *V. cholerae* virulence gene expression (17, 27, 43). Mutation of *V. cholerae hns* causes derepression of *toxT*, *tcpA*, and *ctx* expression to near-wild-type levels even in the absence of cognate activator proteins (43). A variety of data indicate that H-NS directly influences transcription at each of these promoters. Thus, H-NS functions at multiple promoters within the cascade to reduce virulence gene expression under particular environmental conditions.

In the present study, we show that, in contrast to H-NS, the nucleoid protein IHF positively influences virulence gene expression in *V. cholerae*. Deletion of either the *ihfA* or the *ihfB* gene decreased chromosomally encoded *ctx-lacZ* and *tcpA-lacZ* promoter fusion expression and prevented TCP and CT production. The same mutations had no effect on *tcpP-lacZ* or *toxT-lacZ* fusions. Gel mobility shift assays and DNase I footprinting experiments with purified *V. cholerae* IHF demonstrate that IHF recognizes a putative IHF consensus site within the *tcpA* promoter and introduces a bend upon binding. DNase I footprinting at *tcpA* revealed that H-NS binding covers the identified IHF consensus site as well as the ToxT site. In addition, we determined that the dependence on IHF for optimal *tcpA* expression requires the presence of both H-NS and ToxT.

MATERIALS AND METHODS

Bacterial strains and growth. The *V. cholerae* and *E. coli* strains and plasmids used in this study are listed in Table 1. Strains were maintained at −70°C in Luria-Bertani (LB) medium (36) containing 30% (vol/vol) glycerol. Antibiotics were used at the following concentrations in LB medium: ampicillin (Amp), 100 μg/ml; gentamicin, 30 μg/ml; kanamycin (Kan), 45 μg/ml; polymyxin B, 50 IU/ml; and streptomycin, 1 mg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used in LB agar at 40 μg/ml (Sigma).

Construction of chromosomal *ihf* deletions. The *ihfA* subunit deletion was constructed in pKAS32 (53) by PCR using primer HMEco (oligonucleotide sequences are listed in Table 2) with HMNot1 and HMNot2 with HMXba. A Kan^r fragment was inserted between the two fragments at the NotI site, and the resulting plasmid, pGKK158, was used for allelic exchange. The *ihfB* subunit deletion construct was created in pKAS46 (53) by PCR using primer HBEco with HBNotI and primer HBNot2 with HBXba. A Gen^r fragment was inserted between the two fragments at the NotI site, and the resulting plasmid, pGKK162, was used for allelic exchange. Deletions were confirmed by PCR.

Expression plasmids. Plasmid pKAS161 was constructed by PCR amplifying a 1-kb fragment containing the *ihfA* subunit with primers HMEco and HMXba. The resulting product was directly cloned into pBAD-TOPO (Invitrogen) and sequenced.

The *V. cholerae hns* expression plasmid pEAS10 was constructed by PCR amplifying the 414-bp *hns* gene from O395 chromosomal DNA by using HNS1 and HNS2. The resulting fragment was digested with NdeI and SapI and ligated into similarly cut pTXB-1 to generate pEAS10. The nucleotide sequence was confirmed by DNA sequencing.

Construction of IHF binding site mutations. The 21-bp deletion of the putative IHF consensus site in the *tcpA* promoter was constructed using primer pairs H-Eco/Sap1B and ABgl2/Ear1T to amplify chromosomal sequences from O395. The fragments were digested with SapI and EarI to produce seamless junctions, followed by BglII and EcoRI digestion and ligation into pKAS32 to generate pEAS9. The resulting plasmid was confirmed by sequencing. To construct four base pair mutations (shown in Fig. 4C) in the most highly conserved residues of the IHF consensus site, the primer pairs H-Eco/Sap2B and Ear3T/ABgl2 were used to amplify O395 chromosomal DNA. After digestion as described above, the fragments were ligated into pKAS32, generating p4PM-*tcpA*, and sequenced. These mutations were introduced into the *tcpA-lacZ* fusion strain (MBN135) or O395 by allelic exchange and subsequently confirmed by sequencing.

β-Galactosidase assays. β-Galactosidase assays (36) were carried out after overnight growth in LB medium with a starting pH of 6.5 at 30°C and shaking. Due to TCP-mediated bacterial autoagglutination, the specific activities of strains in the *ctx-lacZ* background were calculated using the protein concentration determined by a bicinchoninic acid procedure (Pierce) rather than using the more standard optical density normalization method.

CT assays. GM_1 ganglioside enzyme-linked immunosorbent CT assays (23) were carried out on the supernatants of overnight cultures grown in LB medium with a starting pH of 6.5 at 30°C and shaking.

Immunoblot analysis. Whole-cell protein extracts prepared from the overnight cultures used for CT assays were subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, probed with an anti-TcpA antibody (59) or an anti-ToxT polyclonal antibody, and visualized using an enhanced chemiluminescence detection system (Amersham).

Purification of the *V. cholerae* IHF heterodimer. Plasmids pEAS2 and pEAS3 were generated to overexpress the genes encoding the IHFα and IHFβ subunits, respectively. The *ihfA* gene was amplified from O395 chromosomal DNA by using primers ihfA1 and ihfA2, digested with EcoRI and SphI, and ligated into similarly cut pBAD22, creating pEAS2. The *ihfB* gene was amplified from O395 chromosomal DNA with the primer pair ihfB1/ihfB2, digested with EcoRI and SphI, and ligated into pEAS1, creating pEAS3. pEAS1 was generated by ligating the 50-bp ribosomal binding site from pBAD22 (NheI/SphI-digested fragment) into pKAS178 (a pBAD33 Km^r derivative) to replace the pBAD33 ribosomal binding site. This ensured that the translational efficiencies of *ihfB* from pEAS3 and *ihfA* from pEAS2 were comparable. The nucleotide sequences of both constructs were confirmed by sequencing. *V. cholerae* O395 harboring both pEAS2 and pEAS3 was grown overnight in 10 ml of LB at 37°C with shaking and used to inoculate 2 liters of LB containing Amp/Kan. The culture was grown for 3 h, and arabinose was then added to give a final concentration of 0.1%. Following overnight incubation at 37°C with shaking, the *V. cholerae* IHF heterodimer was extracted as soluble protein as previously described (40). Briefly, the culture was centrifuged, sonicated, and recentrifuged and the supernatant was precipitated with 50% ammonium sulfate. After centrifugation, the resulting

TABLE 1. Bacterial stains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
V. cholerae		
O395 Sm	Classical Ogawa; Sm^r	60
CG842	O395; Δ*lacZ*	8
KSK218	CG842; *ctx-lacZ*; Sm^r Cm^r	54
GK628	KSK218; Δ*ihfA*::*kan*	This work
GK646	KSK218; Δ*ihfB*::*gen*	This work
KSK618	CG842; *tcpP-lacZ*	56
GK630	KSK618; Δ*ihfA*::*kan*	This work
GK652	KSK618; Δ*ihfB*::*gen*	This work
MBN032	CG842; *toxT-lacZ1*	43
GK632	MBN032; Δ*ihfA*::*kan*	This work
GK663	MBN032; Δ*ihfB*::*gen*	This work
MBN135	CG842; *tcpA-lacZ*	43
GK648	MBN135; Δ*ihfA*::*kan*	This work
GK650	MBN135; Δ*ihfB*::*gen*	This work
ES23	MBN135; Δconsensus at *tcpA*	This work
ES24	MBN135; 4PM mutation in *tcpA* IHF consensus site	This work
MBN142	MBN135; Δ*toxT*	43
ES41	MBN135; Δ*toxT* Δ*ihfA*::*kan*	This work
MBN148	MBN135; Δ*hns*	43
ES42	MBN135; Δ*hns* Δ*ihfA*::*kan*	This work
MBN168	MBN135; Δ*toxT* Δ*hns*	43
ES43	MBN135; Δ*toxT* Δ*hns* Δ*ihfA*::*kan*	This work
ES21	O395; Δ*ihfA*::*kan*	This work
E. coli ER2566	Expression strain; T7 RNA polymerase	New England Biolabs
Plasmids		
pKAS32	pGP704; *rpsL*; Ap^r	53
pGKK158	pKAS32; Δ*ihfA*::*kan*; Ap^r Km^r	This work
pKAS46	pKAS32; Km^r	53
pGKK162	pKAS46; Δ*ihfB*::*gen*; Km^r Gm^r	This work
pBAD-TOPO	Expression vector; Km^r	Invitrogen
pKAS161	pBAD-TOPO; *ihfA*; Km^r	This work
pKAS178	pBAD33; Km^r Cm^r	24
pBAD22	Expression vector; Ap^r	19
pEAS1	pKAS178; RBS from pBAD22; Km^r	This work
pEAS2	pBAD22; *ihfA*; Ap^r	This work
pEAS3	pEAS1, *ihfB*; Km^r	This work
pBlueScript	Cloning vector; Ap^r	Stratagene
pEAS4	pBlueScript; 232-bp *tcpA* promoter fragment	This work
pEAS5	pBlueScript; 360-bp *tcpA* promoter fragment	This work
pBEND5	Vector for circular permutation assay; Ap^r	72
pEAS7	pBEND5; 66-bp *tcpA* IHF consensus site	This work
pEAS9	pKAS32; 21-bp deletion including *tcpA* IHF consensus site (Δconsensus)	This work
pTXB-1	Expression vector for intein/chitin binding domain fusion; Ap^r	New England Biolabs
pEAS10	pTXB-1; *hns* Ap^r	This work
p4PM-*tcpA*	pKAS32; *tcpA* IHF consensus 4-point mutations (4PM)	This work
pRH81	pBAD22; His_6 *toxT*	22

supernatant was precipitated with 70% ammonium sulfate and the pellet was resuspended in 1 ml of TG (50 mM Tris [pH 7.4], 10% glycerol) containing 10 mM KCl. The protein was then dialyzed overnight against TG containing 10 mM KCl, loaded onto a phosphocellulose column, and eluted with a KCl salt gradient from 10 mM to 1.2 M in TG. The IHF-containing fractions were pooled, dialyzed against a 25 mM NaH_2PO_4 (pH 5.5), 1 mM EDTA, 5% glycerol buffer, fractionated over a cation exchange Macro-Prep High S support (Bio-Rad) at pH 5.5, and developed with a linear gradient (75 to 800 mM) of NaCl in 25 mM NaH_2PO_4 buffer. The fractions were analyzed on an 18% urea polyacrylamide gel, and the IHF-containing fractions were pooled and further dialyzed against 25 mM NaH_2PO_4, 1 mM EDTA, 5% glycerol. The estimated purity of the IHF heterodimer was 95% as determined by densitometry.

Purification of *V. cholerae* H-NS. H-NS was purified using the IMPACT-CN system, which uses a cleavable chitin binding domain fused to the C terminus of a protein for purification utilizing a chitin column (New England Biolabs). *E. coli* strain ER2566, containing the *hns* expression plasmid pEAS10, was grown in LB containing Amp at 37°C for 3 h, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and grown overnight at 16°C. The cells were collected by centrifugation and resuspended in column buffer (20 mM Tris [pH 8.0], 500 mM NaCl, 1 mM EDTA). The extract was sonicated and clarified by centrifugation at 14,000 rpm for 30 min in an SS34 rotor. The supernatant was then loaded onto a chitin column equilibrated with column buffer to bind the fusion protein and washed with 10 volumes of column buffer and high-salt column buffer (containing 1 M NaCl). Three volumes of cleavage buffer (column buffer containing 100 mM dithiothreitol [DTT]) were added, and the column was incubated overnight at 4°C. H-NS was then eluted off the column with column buffer. The eluted protein was dialyzed overnight in a solution of 20 mM Tris (pH 8.0), 1 mM EDTA, 5 mM potassium glutamate, and 0.1 mM DTT. Glycerol was added to the protein to 10%, and the protein was stored at −70°C. The final purity of H-NS was estimated to be greater than 90% as determined by densitometry.

TABLE 2. Oligonucleotides used in this study

Name	Sequence[a]
HMEco	GATCGGAATTCCAGCGGTAGAGGCGATTGTG
HMNot1	GATCGGCGGCCGCAAAACTTCCCTCAAAGCTAAG
HMNot2	GATCGGCGGCCGCGACCGAGCAATAGACCACGC
HMXba	GATCGTCTAGATTTAGCCCGAGCCACCATAG
HBEco	GATCGGAATTCCGTGACCGTATCGAAGATGC
HBNot1	GATCGGCGGCCGCTAGTTTCCCTCTTCGAGTTG
HBNot2	GATCGGCGGCCGCTCTGAGTAAAGTTTGTTCAC
HBXba	GATCGTCTAGATCTTGCTGGGCGCTACGATG
H-Eco	GATCGGAATTCCACGTTATCAAACATTGCCAG
Sap1B	GATCGGCTCTTCGCTTGTAATTTAATGCAAATAAAATGC
ABgl2	GATCGAGATCTGCTGTCGCATCAGCTGTTGC
Ear1T	GATCGCTCTTCGAAGTTGGCCTTTTTTTAGTGTC
Sap2B	GATCGGCTCTTCG**ACC**TTTAAAGTAATTTAATGCAA
Ear3T	GATCGCTCTTCG**GGT**TTTGAA**G**TGAATAAGTTGGC
ihfA1	GATCGGAATTCACCATGGCGCTCACAAAGGCCGAATTG
ihfA2	GATCGGCATGCGTCTTATTTTTCGACTTTGATG
ihfB1	GATCGGAATTCACCATGACTAAGTCCGAACTGATAG
ihfB2	GATCGGCATGCGATTACAAGTTAACGCGTTCG
HNS1	GATCGCATATGGTAATGTCGGAAATCACTAAG
HNS2	GATCGGCTCTTCAGCACAGAGCGAATTCTTCCAGAGA
TCP-SAL	GACTCGTCGACAATTTCGATCTCCACTCCGG
TCP-XHO	GTCAACTCGAGCATATTTATGTAACTCCACC
ctxP2	GATCGGAATTCTACCGGATTTTAATCACTTTG
ctxP1	GATCGGGATCCCATCAGGAGGTCTAGAATCTG
tcp1	GACTCTCTAGAAGTGTCTGATTTATTTTGTG
tcp2	GACTCGGATCCTTGCTGTGTTTTTTTATTTT
tcp7	GATCGGAATTCATCCACGTAGGTGGGTATAG
tcp9	GATCGGGATCCCATATTTATGTAACTCCAC
tcp12	GCATTTTATTTGCATTAAATTAC
tcp13	GACACTAAAAAAAGGCCAAC

[a] All primer sequences are shown 5′ to 3′. Underlines indicate sequences encoding relevant restriction sites, while substitutions are in bold.

Gel mobility shift assays. DNA fragments containing the putative IHF binding sites in the promoter regions of the *tcpA* and *ctx* operons were generated by PCR from *V. cholerae* O395 chromosomal DNA or from the pEAS9 or p4PM-*tcpA* plasmid with the TCP-SAL (−226)/TCP-XHO (+78), TCP-SAL/tcp2 (−50), tcp1 (−100)/TCP-XHO, or ctxP2 (−194)/ctxP1 (+118) oligonucleotide pair. IHF gel mobility shift assays were carried out in a volume of 20 μl containing DNA-binding buffer (50 mM Tris [pH 7.4], 70 mM KCl, 1 mM EDTA, 1 mM DTT, 100 μg/ml bovine serum albumin, 5% glycerol), 10 ng of digoxigenin (DIG)-labeled DNA, 1.5 μg of calf thymus DNA, and purified IHF heterodimers. After incubation at 30°C for 15 min, protein-DNA complexes were separated from free probes by electrophoresis on 5% nondenaturing polyacrylamide gels and transferred to nylon. H-NS gel mobility shift assays were carried out in a volume of 20 μl containing DNA-binding buffer (40 mM HEPES, 100 mM potassium glutamate, 10 mM magnesium aspartate, 0.022% NP-40, 100 μg/ml bovine serum albumin, 10% glycerol), 10 ng of DIG-labeled DNA, 1 μg of calf thymus DNA, and purified H-NS. After incubation at room temperature for 20 min, protein-DNA complexes were separated by 5% nondenaturing polyacrylamide gel electrophoresis and transferred to nylon. DNA was visualized with an anti-DIG detection kit (Roche), followed by enhanced chemiluminescence detection.

DNase I footprinting. DNA fragments of 232 and 360 bp were generated from the O395 *tcpA* promoter by amplification with the tcp7 (−282)/tcp2 (−50) or tcp7/tcp9 (+78) primer pair. These fragments were ligated into pBlueScript (Stratagene), generating pEAS4 and pEAS5, respectively. Fragments for labeling were digested from pEAS4 and pEAS5 with EcoRI and BamHI. Strand labeling was carried out essentially as described by Kovacikova and Skorupski (26). Binding reactions were performed in a 6.5-μl volume containing ^{33}P-end-labeled DNA, purified IHF or H-NS, and the corresponding binding buffer as described above. After incubation at 30°C for 15 min, protein-DNA complexes were digested with 1 μl of various dilutions of DNase I (Promega) for 5 min at 30°C. The reactions were stopped by addition of 2 mM EGTA, followed by heat inactivation at 65°C for 10 min and then spot dialysis for 30 min against 10 mM Tris (pH 8.0), 0.1 mM EDTA. The reaction mixtures were then heated to 90°C for 5 min in formamide loading buffer (Epicenter) and separated on a 6% acrylamide sequencing gel with 1× Tris-borate-EDTA. The gels were dried and visualized by autoradiography.

Circular permutation assay. The ability of IHF to bend DNA at its binding site was tested as described by Wu and Crothers (66). A 67-bp fragment containing the IHF binding site was amplified with primers tcp12 and tcp13 and cloned into the HpaI site of pBEND5 (72), generating pEAS7. The insert was confirmed by sequencing. Permutated DNA fragments were generated from pEAS7 by digestion with the enzymes listed in Fig. 5, and the resulting 190-bp fragments were purified from polyacrylamide gels and labeled with DIG. A standard gel mobility shift assay with purified IHF was performed on each fragment as described above.

RESULTS

IHF positively regulates expression of the *tcpA* and *ctx* promoters. To assess the influence of IHF on the regulation of virulence gene expression in *V. cholerae*, Δ*ihfA*::*kan* and Δ*ihfB*::*gen* mutants were constructed in the *ctx-lacZ* fusion strain KSK218 (54). Under conditions known to induce virulence gene expression, LB with a starting pH of 6.5 at 30°C, both *ihf* mutants showed fivefold reductions in *ctx-lacZ* expression (Fig. 1A). Since *tcpA* expression is coordinate with that of *ctx*, the influence of the *ihf* mutations on a *tcpA-lacZ* fusion strain, MBN135 (43), was assessed and expression was also reduced approximately fourfold (Fig. 1A). These results show that IHF plays a role in the positive activations of the *ctx* and *tcpA* promoters. Expression of the α subunit of IHF in pBAD-TOPO (pKAS161) in the *ihfA* mutant strain restored normal *ctx-lacZ* and *tcpA-lacZ* expression, confirming that the decreased expression levels observed at these promoters were due to the lack of functional IHF in the Δ*ihfA*::*kan* strain (Fig. 1B).

Introduction of the Δ*ihfA*::*kan* mutation into wild-type O395 confirmed that the production of TCP and CT in *V. cholerae* is dependent upon IHF. As observed in Fig. 2A, the Δ*ihfA*::*kan* mutant ES21 is considerably reduced for TcpA and CT production when grown under inducing conditions. In addition, both of these products were restored with *ihfA* overexpression from pEAS2 (Fig. 2A).

The influence of IHF on virulence gene expression could be the result of a direct effect at the *ctx* and *tcpA* promoters, or the effect could be indirect due to an influence of IHF at promoters further upstream in the virulence cascade, such as the *tcpPH*, *toxR*, or *toxT* promoter. To address the IHF effect on *tcpPH*, the Δ*ihfA*::*kan* and Δ*ihfB*::*gen* mutations were introduced into the *tcpPH-lacZ* strain KSK618 (56). The expression of *tcpPH* was not influenced by either *ihf* mutation (data not shown). Levels of ToxR, a member of the protein pair that cooperates with TcpP/H to activate *toxT* expression, were also unaffected by the Δ*ihfA*::*kan* mutation, as detected by immunoblot analysis (data not shown). The expression of *toxT* is influenced by two promoters, one that is TcpP/ToxR dependent, located immediately upstream of *toxT*, and one that is ToxT dependent and initiates upstream of *tcpA* (see Fig. 8). To determine the effect of IHF on *toxT* expression from the TcpP/ToxR-dependent promoter, the Δ*ihfA*::*kan* and Δ*ihfB*::*gen* mutations were introduced into the *toxT-lacZ* strain MBN032, which is lacking ToxT (43). Expression from this promoter was not influenced by either *ihf* mutation (data not shown). To address whether IHF influences *toxT* expression from the ToxT-dependent promoter upstream of *tcpA*, a Western blot analysis was performed to assess whether total ToxT levels were altered in the *ihfA* mutant. As shown in Fig. 2B, ToxT levels were lower in the *ihfA* mutant than in the wild type upon growth to late log phase under inducing conditions. Additionally, when total *toxT* transcript from nonfusion backgrounds was measured by quantitative real-time PCR from cultures

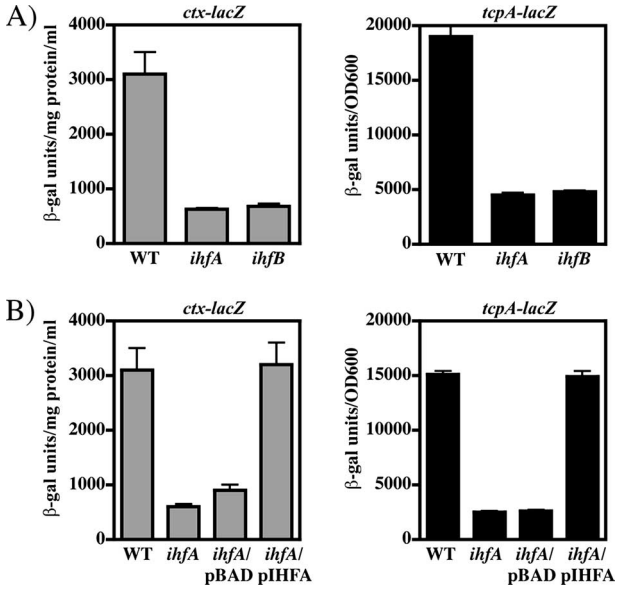

FIG. 1. Influence of IHF on *ctx-lacZ* and *tcpA-lacZ* expression. (A) Wild-type (WT) (*tcpA-lacZ* or *ctx-lacZ*), *ihfA* (Δ*ihfA*::*kan*), or *ihfB* (Δ*ihfB*::*gen*) mutants. (B) WT, *ihfA*, or *ihfA* mutants with the empty vector pBAD-TOPO or the IHFα expression vector pIHFA (pKAS161). Cultures were grown overnight in LB medium with a starting pH of 6.5 at 30°C with shaking (those with plasmids contained 0.1% arabinose) and assayed for β-galactosidase production. The values are the averages for at least two independent experiments.

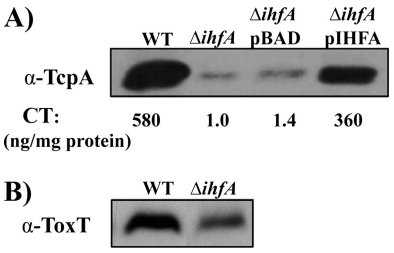

FIG. 2. TcpA, CT, and ToxT production in O395 and the *ihfA* mutant. Strains were grown overnight in LB medium with a starting pH of 6.5 at 30°C with shaking. Western blot analysis with the anti-TcpA (α-TcpA) antibody (A) or α-ToxT polyclonal antibody (B) was performed with 16 μg of total protein extract. CT represents the amount of CT detected in the culture supernatant as measured by a GM_1-ganglioside enzyme-linked immunosorbent CT assay (A) (expressed in ng/mg of protein). WT, wild type.

grown overnight under inducing conditions, *toxT* transcript levels were decreased similarly to *tcpA* transcript levels in the *ihfA* mutant compared to wild-type levels (data not shown). The observation that IHF does not appear to influence expression from the TcpP/ToxR-dependent promoter and yet total transcript levels (TcpP/ToxR-dependent and ToxT-dependent *toxT* transcripts) were reduced in the *ihfA* mutant suggests that IHF might have a direct effect on the ToxT-dependent promoter upstream of *tcpA*. The decreases in *ctx* expression and CT production in this mutant are most likely due to reduced *toxT* expression resulting from the influence of IHF at the *tcpA* promoter. This is further supported by studies of IHF binding at these promoters (see below).

Purified IHF from *V. cholerae* binds the *tcpA* promoter region between positions −226 and −50. Gel mobility shift assays were used to assess whether IHF directly binds to the virulence gene promoter regions. Increasing amounts of purified *V. cholerae* IHF (180 and 360 nM) were incubated with 10 ng of *ctx* (C) or *tcpA* (A1) promoter fragments. IHF did not bind the *ctx* fragment (C) but did bind the *tcpA* fragment (A1) (Fig. 3), suggesting a direct role for IHF at the *tcpA* promoter and not the *ctx* promoter. *tcpP* and *toxT* promoter fragments also failed to bind IHF in gel mobility shift assays (data not shown). These results are consistent with expression data that suggest that IHF does not have a direct regulatory role at the *tcpP* or *toxT* promoters.

To locate the IHF binding site within the *tcpA* promoter region, smaller fragments (A2 and A3) were used to determine where binding occurs. Two putative IHF consensus sites based on the *E. coli* IHF consensus site WATCAANNNNTTR, where W represents A or T and R represents A or G, are present within the *tcpA* promoter. One site overlaps the −35 region (5′-CATCTGTCAATTG), while the second is located upstream and centered at position −162 (5′-AATCATTTGA ATT), about 80 bp upstream of the ToxT binding site (positions −84 to −41). IHF was capable of binding to the promoter fragment containing the −162 site alone (−226 to −50) (A2), whereas it was not capable of binding to the −35 site alone (−100 to +78) (A3) (Fig. 3). Despite the presence of only one shifted complex on the A1 fragment containing both putative sites, it is formally possible that IHF binds the −35 site only in the presence of the −162 site. This possibility was examined in DNase I footprinting experiments where both sites were present on the same fragment (see below). Notably, the DNA surrounding the −162 site from position −200 to −125 has 76% AT content. The high AT content, coupled with the knowledge that the nucleoid-associated protein H-NS directly represses *tcpA* expression (71) and itself preferentially binds AT-rich DNA (18, 32, 42, 44), suggests that the region around position −162 may also be an H-NS binding site. Scanning of this region for putative H-NS nucleation sites by use of the 10-bp consensus site identified by Lang et al. (29) revealed two matches adjacent to the IHF consensus site (Fig. 4C, boxes), further suggesting that IHF and H-NS could both bind this region of the *tcpA* promoter.

IHF and H-NS binding sites overlap the −162 IHF consensus site. DNase I footprinting was used to determine if IHF and H-NS bind the same −162 region within the *tcpA* promoter. Radiolabeled fragments that spanned the *tcpA* promoter region from position −282 to −50 (IHF footprint) and from position −282 to +78 (H-NS footprint) were generated, incubated with increasing amounts of IHF (1.2 μM and 2.4 μM) and H-NS (1 μM, 2 μM, and 4 μM), respectively, and digested with DNase I as described in Materials and Methods. The reaction mixtures were then subjected to electrophoresis on a denaturing polyacrylamide gel. IHF protected a region from position −185 to −126 on the top strand and a region from position −198 to −136 on the bottom strand (Fig. 4A). This protection overlapped the putative IHF consensus site at position −162 and the two putative H-NS nucleation sites, as shown in Fig. 4C. DNase I-hypersensitive sites, a characteristic of DNA bending, flank the IHF protection, suggesting that IHF might be bending the *tcpA* promoter upon binding. IHF

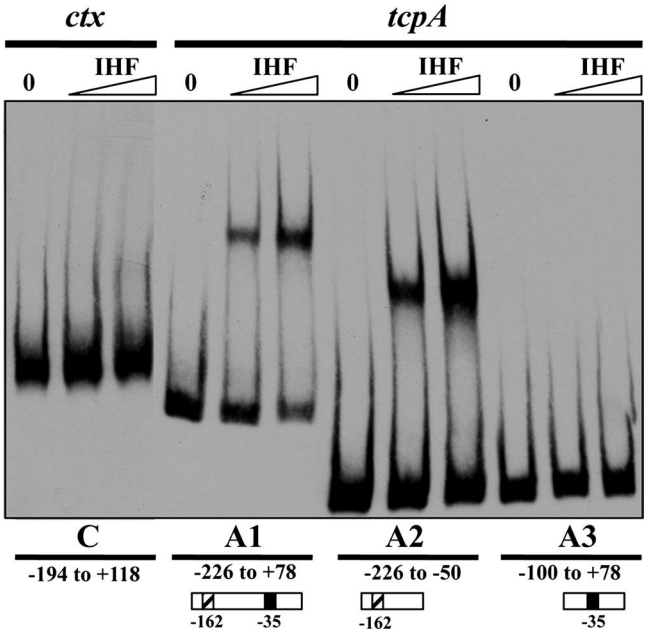

FIG. 3. Gel mobility shift assay for IHF binding to *ctx* and *tcpA* promoter DNA. The DIG-labeled promoter fragments of *ctx* from position −194 to +118 (C) and *tcpA* from position −226 to +78 (A1), −226 to −50 (A2), and −100 to +78 (A3) were incubated with purified IHF and analyzed by a gel mobility shift assay. The first lane in each set has no protein added, the second lane contains 180 nM IHF, and the third land contains 360 nM IHF. The boxes labeled −162 and −35 represent putative IHF consensus sites on the *tcpA* promoter fragments.

did not protect the putative IHF consensus site at the −35 site on the longer (−282 to +78) *tcpA* fragment that contained both IHF consensus sites (data not shown). This suggests that the putative IHF site at position −35 is not bound by IHF even in the presence of the −162 site and that the single shifted complex observed with the A1 fragment in Fig. 3 is IHF binding to the −162 site alone.

H-NS binding was more widespread than IHF binding within the *tcpA* promoter region. H-NS bound over the IHF binding site at position −162 and strongly protected at least part of the ToxT binding site that spans from position −84 to −41 in the promoter region (71) (Fig. 4B). This binding region has an AT content >70% similar to that of the IHF binding region. These results suggest that H-NS binding overlaps the binding sites of both activators, IHF and ToxT, at the *tcpA* promoter and could act to block the binding of these activator proteins.

IHF binding induces bending of the DNA upstream of *tcpA*. IHF has been shown to bend DNA in a number of systems (61), and this property has been suggested to be central to its activities (39, 50, 57). We used the circular permutation assay of Wu and Crothers (66) to determine the ability of IHF to bend 190-bp restriction fragments containing a 67-bp fragment of the *tcpA* promoter region encompassing the IHF binding site at different positions relative to the ends of the restriction fragment, as shown in Fig. 5A. The bending assay is based on the observation that differences exist in the migration of linear and bent DNA fragments and that the electrophoretic mobility of bent protein-bound DNA differs with the positioning of the DNA-binding site relative to either end of the fragment. The more centrally located the bending site, the more retarded the migration of the protein-DNA complex. Gel mobility shift assays with each fragment in the presence of IHF were carried out as described above. All free DNA migrated at the same rate, suggesting that there was no intrinsic bend in the DNA. Mobility upon incubation with IHF was slowest when the IHF binding site was centrally located (EcoRV) and maximal when the site was closest to the end of the fragment (BglII and BamHI). These results are consistent with the center of the bend being located at the IHF binding site. Migration of the fragments bound by IHF was measured from the well, and the bending angle was calculated to be 120° by the method of Thompson and Landy (61).

The influence of IHF consensus site mutations on IHF binding and *tcpA* expression. The IHF consensus site was altered in order to disrupt IHF binding and activity at the *tcpA* promoter. The first binding site mutation, termed Δconsensus, was a 21-bp deletion that included the 13-bp IHF consensus site (Fig. 4C). The second, termed 4PM, was the introduction of 4-base-pair changes in the most highly conserved consensus site residues, in which the TCA and the second-to-last T were changed to GGT and G, respectively (Fig. 4C). Fragments of the *tcpA* promoter region from position −226 to +78 containing these mutations were assessed for their effects on IHF binding in gel mobility shift assays as described above. IHF did not shift fragments containing the Δconsensus or the 4PM mutation (Fig. 6A). These results suggest that this site is essential for IHF binding. These mutations were then assayed for their effects on *tcpA* transcription by integration into the *tcpA-lacZ* background. Contrary to what was expected, neither the Δconsensus nor the 4PM mutation mimicked an *ihf* mutant in its effect on *tcpA* expression. Rather than the 4-fold decrease seen previously in the *ihf* mutants, the promoter IHF consensus mutations caused only a 1.6-fold decrease in *tcpA-lacZ* expression (data not shown). In addition, TcpA protein production was similar to wild-type levels when these mutations were placed in the otherwise wild-type O395 background (data not shown). This result suggests that the mechanism of IHF activation at this site may be more complicated than simply binding to the IHF consensus site.

H-NS binding to the IHF consensus mutations in gel mobility shift assays. One of the mechanisms by which IHF can regulate transcription is alleviation of H-NS-mediated repression of transcription (63, 64). Based on the idea that IHF can act as an antirepressor and given the DNase I protection data that show overlapping H-NS and IHF sites, we chose to investigate whether the IHF consensus site mutations affected H-NS binding to the *tcpA* promoter fragments. Gel mobility shift assays were performed with wild-type and mutant *tcpA* promoter fragments from position −226 to +78 with increasing amounts of H-NS (Fig. 6B). At low concentrations of H-NS, two shifted bands occurred with the wild-type fragment, a result consistent with our DNase I protection determination that showed multiple H-NS binding sites within this promoter region. The wild-type fragment was completely shifted in the presence of 320 nM H-NS, whereas fragments containing the Δconsensus and 4PM mutations were not fully shifted at 320 nM H-NS. These results suggest that the IHF consensus site within the *tcpA* promoter not only is critical for IHF binding but also contributes to optimal H-NS binding. The reduced

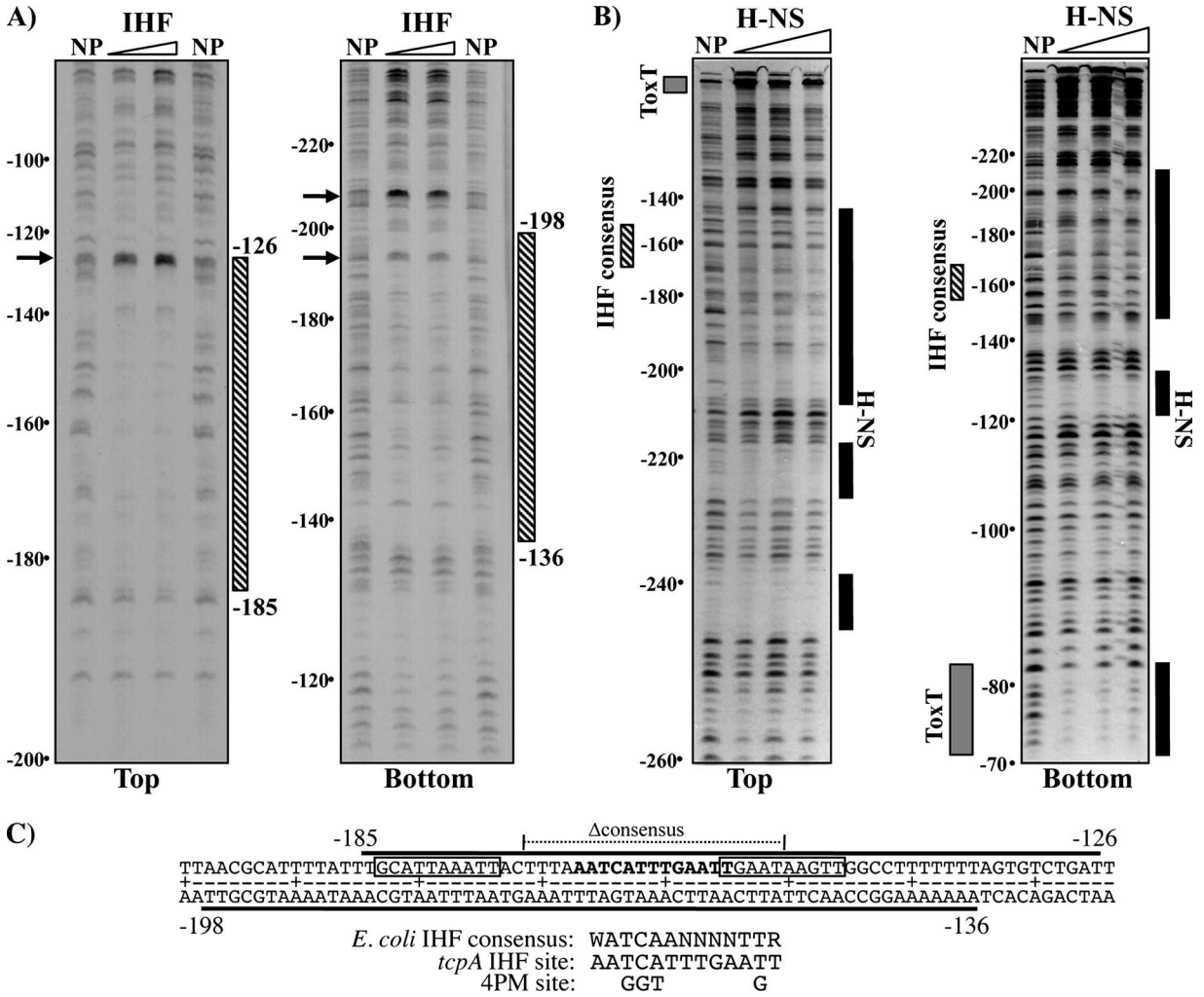

FIG. 4. DNase I protection by IHF and H-NS at the *tcpA* promoter. The top and bottom strands of the *tcpA* promoter fragments were incubated with increasing amounts of IHF (1.2 and 2.4 μM) (A) or H-NS (1, 2, and 4 μM) (B) and treated with DNase I. (A) IHF protection from DNase I on a promoter fragment from position −282 to −50 is shown with hatched bars. Arrows designate sites that were hypersensitive to DNase I treatment. NP, no protein. (B) H-NS protection on a promoter fragment from position −282 to +78 is shown with black bars to the right. On the left, the hatched bars designate the IHF consensus site and the gray boxes designate ToxT binding sites. (C) *tcpA* promoter sequence from position −200 to −125. Black bars designate the IHF protection on the top and bottom strands, and the IHF consensus site within the protected region is in bold and aligned with the *E. coli* consensus site below. W, A/T; R, A/G; N, any nucleotide. The 21 bp that were deleted in the Δconsensus mutation are marked above the sequence. The four base pair mutations that were incorporated into the consensus site are designated 4PM below the consensus alignment. Boxes indicate putative H-NS nucleation sites with seven matches to the 10-bp H-NS consensus site (29).

H-NS binding in the Δconsensus and 4PM mutants may thereby serve to decrease repression of the *tcpA* promoter and partially alleviate the requirement for IHF. The Δconsensus promoter mutation deletes 5 of the 10 bp that comprise the second putative H-NS high-affinity nucleation site (Fig. 4C) and could function to disrupt H-NS nucleation and polymerization from this site.

Competitive gel mobility shift assays were performed between IHF and H-NS on the *tcpA* promoter region to examine the effect of adding either protein to prebound DNA complexes of the other. The DNA was incubated with the lowest concentrations of H-NS or IHF (320 or 420 nM, respectively) that gave a complete shift of the free DNA. After 15 min at 30°C, increasing amounts of IHF or H-NS, respectively, were added and incubation was continued for an additional 15 min. Addition of either 120 or 240 nM of IHF to a prebound H-NS fragment did not influence H-NS binding. However, addition of 420 nM IHF resulted in displacement of H-NS from the DNA and a shift to a predominately IHF-DNA complex. Addition of 160 to 320 nM H-NS to a prebound IHF-DNA fragment did not significantly influence the IHF-DNA complex. These results suggest that IHF effectively displaces H-NS from the *tcpA* promoter fragment and that, once bound, IHF can essentially prevent H-NS binding.

Interplay of IHF, H-NS, and ToxT in the regulation of *tcpA* expression. It is known that H-NS represses *tcpA* transcription. It has been shown that IHF alleviates H-NS repression at the bacteriophage Mu early (Pe) promoter and at the *virB* promoter in *Shigella flexneri* (47, 63). In an attempt to establish whether IHF functions as an antirepressor at the *tcpA* pro-

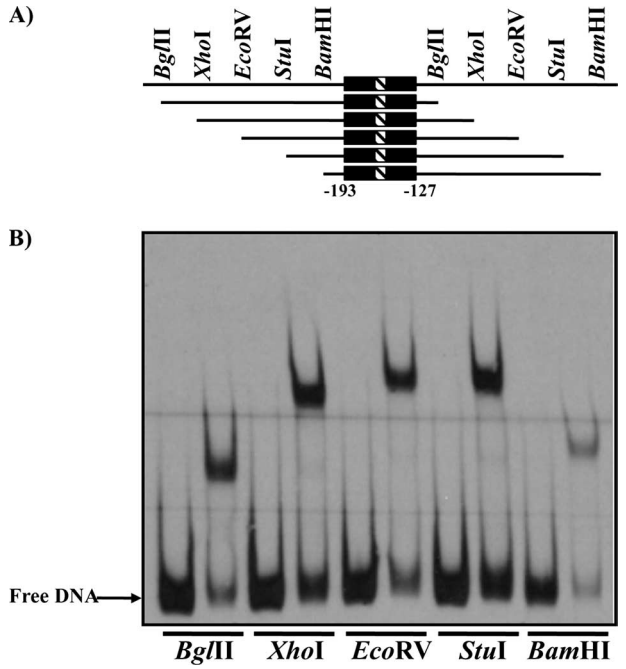

FIG. 5. Bending of the *tcpA* promoter DNA by IHF as determined by a circular permutation assay. (A) Diagram of 190-bp linear probes produced by digestion of plasmid pEAS7 (pBEND5-*tcpA*) with five restriction enzymes. The probes contain the IHF consensus site (hatched box) within a 67-bp *tcpA* promoter fragment (−193 to −127) (black box) at various positions relative to the ends of the fragments. (B) Circular permutation analysis was performed on the *tcpA* IHF binding site with purified IHF. Each fragment was incubated without protein or with 180 nM IHF and analyzed by a gel mobility shift assay. The bending angle, α, was determined to be 120° using the equation $(\mu_M/\mu_E) = \cos(\alpha/2)$, where μ_M is the relative migration of the fragment with the bend center in the middle and μ_E is the relative migration of the fragment with the bend center at the end (61).

moter, we investigated expression levels in strains harboring wild-type or mutant copies of *hns* and *ihfA*. Cultures of the *tcpA-lacZ* strains MBN135 (wild type), GK648 (*ihfA*::*kan*), MBN148 (Δ*hns*), and ES42 (*ihfA*::*kan* Δ*hns*) were grown overnight in LB with a starting pH of 6.5 at 30°C, and β-galactosidase activity was measured. As was previously described by Nye et al. (43), the level of *tcpA-lacZ* expression in the absence of *hns* (MBN148) was increased about 1.5-fold compared to the wild-type (MBN135) level (Table 3). In the absence of *hns* (MBN148), there was no further effect of deleting *ihf* on *tcpA-lacZ* expression, suggesting that the *ihf* mutation is epistatic to the *hns* mutation at the *tcpA* promoter. Furthermore, deleting *hns* has a much greater derepressive effect on *tcpA-lacZ* expression in the absence of *ihfA* than when *ihfA* is present (501% versus 145%) (Table 3). This suggests that IHF counteracts H-NS repression at *tcpA*.

In an attempt to determine if IHF-mediated activation at the *tcpA* promoter is ToxT dependent, we investigated expression levels in strains harboring wild-type or mutant copies of *toxT* and *ihfA*. Cultures of the *tcpA-lacZ* strains MBN135 (wild type), GK648 (*ihfA*::*kan*), MBN142 (Δ*toxT*), and ES41 (*ihfA*::*kan* Δ*toxT*) were grown overnight in LB with a starting pH of 6.5 at 30°C, and β-galactosidase activity was measured. As previously described by Hulbert and Taylor (22), there is a loss of *tcpA-lacZ* expression in the absence of *toxT* (MBN142) compared to that in a *toxT*-containing strain (MBN135) (Table 3). There was no further loss of *tcpA-lacZ* expression in the *ihfA toxT* double mutant (ES41) compared to that in the *toxT* mutant alone, suggesting that the positive effect of IHF at *tcpA* requires ToxT. Furthermore, there was no affect of the *ihf* mutant in an *hns toxT* background (MBN168) (Table 3). Taken together, these results suggest that the IHF effect on *tcpA* expression requires the presence of the repressor H-NS and the activator ToxT.

Given that *toxT* expression is higher in an *hns* mutant due to derepression (43) and that the *ihf* effect requires the presence of ToxT, it is possible that the level of ToxT within the cell determines the necessity for IHF at *tcpA*. We therefore examined the effect of overexpressing *toxT* in the *ihfA*::*kan* background to determine if higher levels of ToxT could overcome the role of IHF in activation. Wild-type O395 or the O395 *ihfA* mutant ES21, harboring an empty vector or the *toxT* overexpression vector pRH81, was grown with or without arabinose to regulate *toxT* expression. Total proteins were extracted, separated, and subjected to Western blotting. The levels of ToxT were slightly reduced in the *ihfA* mutant strain compared to wild-type levels when *toxT* was not induced from the plasmid (Fig. 7), consistent with Fig. 2B. In addition, as seen in Fig. 2A, there is a drastic reduction in TcpA levels in the *ihfA* mutant compared to wild-type levels, despite this modest difference in ToxT levels. In the wild-type strain, overexpression of *toxT* did not detectably increase TcpA production (Fig. 7). However, TcpA was restored to near-wild-type levels upon overexpression of *toxT* in the *ihf* mutant. These results suggest that if ToxT levels are sufficiently high, IHF is not essential for maximal TcpA production. ToxT levels accumulate during exponential growth but decline in stationary phase (data not shown). IHF may be necessary when ToxT levels are low (early exponential phase and stationary phase under virulence gene-inducing conditions) to facilitate ToxT binding and activation at *tcpA* by overcoming some H-NS repression and/or altering promoter structure to facilitate ToxT binding. Similar to *E. coli* IHF (1), *V. cholerae* IHF is produced throughout the growth curve (under both virulence-inducing and -noninducing conditions), but levels are maximal in stationary phase (data not shown). This is consistent with a role for IHF in facilitating ToxT-mediated activation of *tcpA* when ToxT levels are low.

DISCUSSION

In the *V. cholerae* El Tor strain N16961, McLeod et al. have found that, as in *E. coli* and *Salmonella* strains (2, 33), over 100 genes are differentially regulated in an *ihfA* mutant (34). In the work presented here, we show that IHF positively influences the expression of virulence genes in the *V. cholerae* classical O395 strain. The introduction of the *ihfA*::*kan* and *ihfB*::*gen* alleles into the *tcpA-lacZ* (MBN135) and *ctx-lacZ* (KSK218) fusion strains resulted in a four- to fivefold reduction in the expression of *lacZ* and prevented TCP and CT production. A reduction in expression was not observed at the TcpP/ToxR-dependent *toxT* promoter immediately upstream of the *toxT*

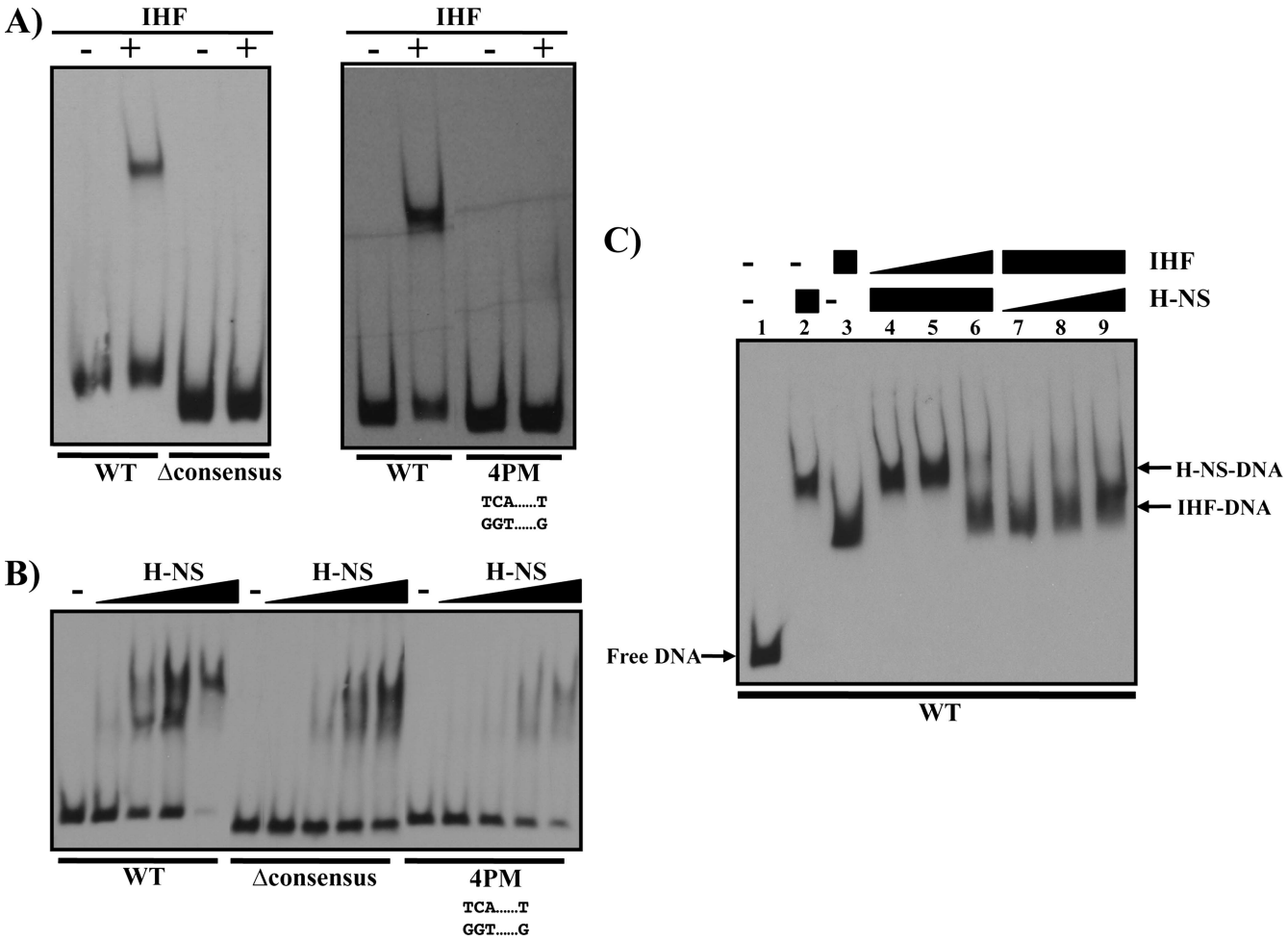

FIG. 6. Mutations in the *tcpA* IHF consensus site alter IHF and H-NS binding. Mutations in the *tcpA* promoter were constructed to disrupt IHF binding. The wild-type (WT) and mutant promoter fragments (Δconsensus and 4PM) from position −226 to +78 were incubated in the absence of protein or with 180 nM IHF (A) or 80, 160, 250, or 320 nM H-NS (B) and analyzed by a gel mobility shift assay. (C) Competitive gel mobility shift assay between H-NS and IHF on the wild-type promoter fragment. Lane 1, no protein added. Lane 2, 320 nM H-NS; lane 3, 420 nM IHF. Lanes 4 to 6, 320 nM H-NS and 120 nM (lane 4), 240 nM (lane 5), and 420 nM (lane 6) IHF. Lanes 7 to 9, 420 nM IHF and 160 nM (lane 7), 250 nM (lane 8), and 320 nM (lane 9) H-NS.

gene (Fig. 8). However, the observation that the levels of ToxT protein in the *ihfA* mutant were lower than those in the wild type suggests that IHF might exert its effects at the ToxT-dependent promoter upstream of *tcpA* that also influences *toxT* expression. A direct role for IHF at the *tcpA* promoter was demonstrated by the ability of the purified protein to bind to a site centered at position −162 in gel mobility shift and DNase I footprinting assays. In addition, the protein was found to bend the DNA 120°. Since IHF did not appear to bind to the *ctx* promoter, these results suggest that the IHF effect on *ctx*

TABLE 3. Effect of IHF on expression of the *tcpA-lacZ* fusion in wild-type, *hns*, *toxT*, and *hns toxT* strains

Strain	β-Galactosidase activity (%)[a]		Fold change
	With *ihfA*	Without *ihfA*	
MBN135 (wild type)	18,591 ± 520 (100)	4,890 ± 270 (100)	3.8
MBN148 (*hns*)	26,956 ± 750 (145)	24,528 ± 526 (502)	1.1
MBN142 (*toxT*)	331 ± 27 (1.8)	355 ± 15 (7.3)	1.1
MBN168 (*hns toxT*)	1,475 ± 84 (7.9)	1,770 ± 93 (36.2)	1.2

[a] β-Galactosidase activities (in Miller units) are shown for each strain and expressed as percentages of the wild-type level. The values are the averages for at least two independent experiments.

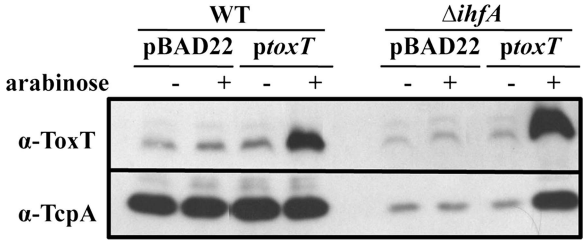

FIG. 7. Overexpression of *toxT* in the *ihfA* mutant background. Strains were grown overnight in LB medium with a starting pH of 6.5 at 30°C with shaking and with the addition of 0.1% arabinose where indicated. Western blot analysis with anti-ToxT (α-ToxT) and α-TcpA antibodies was performed with 16 μg of total protein extract. WT, wild type.

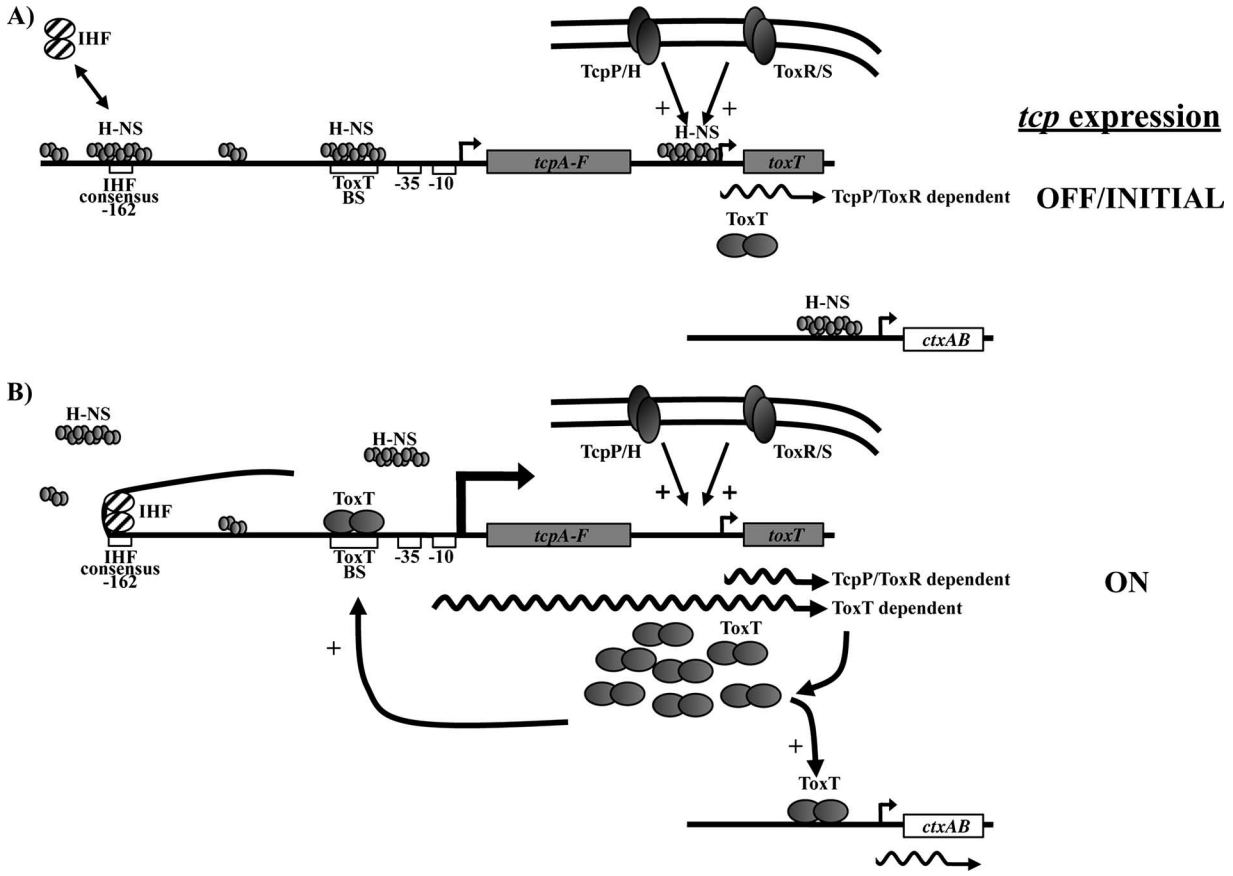

FIG. 8. Schematic of the transcriptional regulation of *V. cholerae tcpA*, *toxT*, and *ctx* expression. (A) OFF/INITIAL: H-NS represses gene expression at multiple promoters within the ToxR regulon, including *tcpA* to *-F*, *toxT*, and *ctx*. H-NS binding in the *tcpA* promoter region overlaps the IHF consensus sequence at position −162 as well as the ToxT binding site spanning from position −84 to −41. (B) ON: After a transition to virulence-inducing conditions, ToxR/S and TcpP/H in the inner membrane cooperate at the *toxT* promoter to activate *toxT* expression (TcpP/ToxR dependent). ToxT-mediated activation of *tcpA-toxT* is facilitated by IHF binding, bending of the DNA, and displacement of H-NS, allowing for greater accessibility of ToxT to its binding site. As ToxT levels accumulate, full activation of *tcpA* to *-F*, *toxT*, and *ctx* expression occurs.

expression is indirect due to lowered *toxT* expression driven from the ToxT-dependent promoter upstream of *tcpA*.

A direct role for H-NS in the repression of *tcpA* expression has previously been described (43, 71). Here, we show direct binding of H-NS to the *tcpA* promoter by gel mobility shift assays and DNase I footprinting. H-NS protection covered the IHF binding site (position −162) as well as the ToxT binding site (which spans from −84 to −41) (Fig. 8A). Genetic studies of *tcpA-lacZ* expression in strains harboring wild-type or mutant copies of *ihfA*, *hns*, or *toxT* showed that activation by IHF appears to occur only when both H-NS and ToxT are present; the *ihfA* mutation is epistatic to the *hns* and *toxT* mutations. The functional dependence of IHF on H-NS and ToxT at *tcpA*, along with the observed overlapping binding sites of these proteins, suggests a possible model whereby IHF functions as an antirepressor at this promoter. This is further supported by the binding studies that show that IHF can displace H-NS from the *tcpA* regulatory region and, once bound, occlude H-NS binding.

As proposed in Fig. 8A, under noninducing conditions, H-NS binds directly to the *tcpA* promoter covering the IHF consensus sequence at position −162 as well as the ToxT binding site (spanning from −84 to −41), thereby repressing the transcription of *tcpA* to *-F*. H-NS also appears to directly repress *toxT* and *ctx* expression (43, 71). Under inducing conditions (Fig. 8B), ToxT levels from the TcpP/ToxR-dependent promoter increase due to cooperation between ToxR/S and TcpP/H in the inner membrane. Activation of the ToxT-dependent *tcpA-toxT* transcript is facilitated by binding of IHF to the site centered at position −162 that introduces a bend in the promoter that partially displaces H-NS from the DNA. This allows greater accessibility of ToxT to its binding site, resulting in transcriptional activation of the *tcpA* to *-F* genes as well as *toxT*. The subsequent increase in ToxT protein levels permits full activation of *tcpA* to *-F* and *ctx* expression. In the absence of IHF, we propose that under inducing conditions, the increased levels of ToxT from the TcpP/ToxR-dependent promoter are insufficient to fully overcome H-NS repression, and thus, expression of *tcpA* to *-F*, *toxT*, and *ctx* is not induced to maximal levels.

Consistent with this model is the finding that loss of the IHF protein results in a fourfold reduction in *tcpA* expression, whereas disruption of the IHF binding site by introduction of the Δconsensus or 4PM mutation, both of which alter IHF and H-NS binding, causes only a small reduction in *tcpA* expression. Because the IHF and H-NS binding sites overlap, these

promoter mutations phenotypically negate each other by simultaneously decreasing *tcpA* expression due to a reduction in IHF-mediated activation and increasing *tcpA* expression due to reduced H-NS repression. The hypothesis that IHF functions as an antirepressor at the *tcpA* promoter is also supported by the finding that IHF is not required upon overexpression of the primary activator ToxT.

There are a number of reports of site-specific DNA-binding proteins acting as H-NS antagonists by displacing H-NS from common or adjacent binding sites. For example, H-NS is displaced by VirB binding at the *icsB* promoter in *S. flexneri* (62), LeuO binding at the *ompS1* promoter in *S. enterica* (11), and SlyA binding to two distal SlyA/H-NS binding sites at *hlyE* in *E. coli* K-12 (31). At the *yjjQ-bglJ* promoter in *E. coli*, LeuO-induced DNA looping is proposed to prevent H-NS oligomerization along the promoter (58). Similarly, IHF may function at *tcpA* to displace H-NS from their overlapping binding site, and the bend introduced upon IHF binding may prevent oligomerization along the promoter. A role for IHF in overcoming H-NS repression at the early promoter (Pe) of bacteriophage Mu has already been established (63). The Pe promoter of bacteriophage Mu appears to be specifically inhibited by the binding of H-NS to the promoter region. Binding of IHF to the upstream site within the Pe promoter results in direct stimulation of the promoter and also interferes with the formation of DNA-protein complexes between H-NS and Pe, thereby counteracting H-NS repression of this promoter. IHF also alleviates H-NS repression at the *virB* promoter in *S. flexneri* (47). In this example, IHF binding does not overlap the H-NS binding site as in the case of the Pe promoter of Mu but is believed to generate a conformational change in the DNA from an upstream location that facilitates the binding of the AraC-like activator VirF, which itself overcomes H-NS repression. Similarly, ToxT appears to function, at least in part, to alleviate H-NS repression at the *tcpA* promoter, in addition to its role as an activator, since there is less H-NS repression of *tcpA* in the presence of ToxT than in its absence (1.4-fold [wild type versus Δ*hns*] versus 4.5-fold [Δ*toxT* versus Δ*toxT* Δ*hns*]).

Many bacterial pathogens have acquired virulence genes by horizontal gene transfer, and it has been suggested that gene silencing by H-NS allows the newly acquired genes to be integrated into regulatory networks, like the ToxR regulon in *V. cholerae*, over time without initially negatively affecting the fitness of the recipient bacterium (14). Enteric bacteria, like *V. cholerae*, encounter multiple environmental stimuli both inside and outside the human host. It is not surprising, therefore, that bacteria have evolved complex regulatory systems to overcome H-NS silencing and respond to a range of environmental inputs. As far as we know, the primary role of TCP and CT is in human infection. The role of the global regulators H-NS and IHF in infection has been hard to define due to their pleiotropic effects on transcription and the growth defects associated with their loss. It is likely that H-NS and IHF function to modulate the topology of the promoters on which the more specific regulators, like ToxT, can act to optimize virulence gene expression in *V. cholerae*. Using microarray analysis, Xu et al. observed that *hns* is one of the most highly expressed *V. cholerae* genes in vitro and in the rabbit ileal loop model of *V. cholerae* infection (67). Additionally, Ghosh et al. (17) reported that H-NS represses *ctx* and *tcpA* expression in vivo (17). This group saw that the in vivo expression levels of *ctx* and *tcpA* were increased in an *hns* mutant 8 h after infection of the rabbit ileal loop. In addition, they noted that CT production was about 2.5-fold greater in the *hns* mutant than in the wild-type O395 strain. These data demonstrate that H-NS is highly expressed and active as a repressor at the *ctx* and *tcpA* promoters in this in vivo model. Proper colonization most likely requires a mechanism to counteract some of this H-NS repression in order to achieve optimal virulence gene expression and cause disease. Yu and DiRita (71) showed that ToxT-mediated activations of the *ctx* and *tcpA* promoters are mechanistically distinct. At *ctx*, ToxT has a higher affinity for its binding site than it does at *tcpA* (71). However, Lee et al. have shown that during infection, maximal *tcpA* expression occurs before maximal *ctx* expression (30). This is likely due, in part, to the greater H-NS repressive effect on *ctx* expression than on *tcpA* expression (43, 71). The differential expression might also be due to the action of IHF at *tcpA* that could serve to lower the threshold level of ToxT that is required to activate *tcpA* expression despite the lower affinity of ToxT for its binding site at this promoter. Further analysis of the molecular interactions between IHF, H-NS, and ToxT at the *tcpA* promoter will provide additional insights into the mechanism by which these proteins interact to influence the later steps in the cascade that regulates virulence gene expression in *V. cholerae*.

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