8-2000

Vibrio cholerae H-NS Silences Virulence Gene Expression at Multiple Steps in the ToxR Regulatory Cascade

Melinda B. Nye
Dartmouth College

James D. Pfau
Dartmouth College

Karen Skorupski
Dartmouth College

Ronald K. Taylor
Dartmouth College

JOURNAL OF BACTERIOLOGY, Aug. 2000, p. 4295–4303 Vol. 182, No. 15
0021-9193/00/$04.00+0

Vibrio cholerae H-NS Silences Virulence Gene Expression at Multiple Steps in the ToxR Regulatory Cascade

MELINDA B. NYE, JAMES D. PFAU, KAREN SKORUPSKI, AND RONALD K. TAYLOR*

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755

Received 12 January 2000/Accepted 15 May 2000

H-NS is an abundant nucleoid-associated protein involved in the maintenance of chromosomal architecture in bacteria. H-NS also has a role in silencing the expression of a variety of environmentally regulated genes during growth under nonpermissive conditions. In this study we demonstrate a role for H-NS in the negative modulation of expression of several genes within the ToxR virulence regulon of *Vibrio cholerae*. Deletion of *hns* resulted in high, nearly constitutive levels of expression of the genes encoding cholera toxin, toxin-coregulated pilus, and the ToxT virulence gene regulatory protein. For the cholera toxin- and ToxT-encoding genes, elevated expression in an *hns* mutant was found to occur in the absence of the cognate activator proteins, suggesting that H-NS functions directly at these promoters to decrease gene expression. Deletion analysis of the region upstream of *toxT* suggests that an extensive region located far upstream of the transcriptional start site is required for complete H-NS-mediated repression of gene expression. These data indicate that H-NS negatively influences multiple levels of gene expression within the *V. cholerae* virulence cascade and raise the possibility that the transcriptional activator proteins in the ToxR regulon function to counteract the repressive effects of H-NS at the various promoters as well as to recruit RNA polymerase.

Vibrio cholerae is the bacterial causative agent of the acute diarrheal disease called cholera. The organism is spread among individuals through the ingestion of contaminated water or food. In areas where cholera is endemic, the organism persists in an aquatic niche between periodic outbreaks. In the human host, *V. cholerae* pathogenesis involves the coordinated expression of a number of virulence factors, including cholera toxin (CT), which is directly responsible for the disease symptoms, and toxin-coregulated pilus (TCP), which is required for intestinal colonization. Expression of the genes that encode these virulence factors is regulated at the transcriptional level by a variety of parameters, such as osmolarity, temperature, pH, anaerobiosis, and chemoattractant amino acids (16, 30). Such regulation is thought to provide a mechanism by which the organism can induce the expression of virulence genes within the host and repress them under growth conditions where they are not required.

Transcriptional regulation of the genes encoding CT and TCP occurs via a cascade involving several activator proteins referred to collectively as the ToxR virulence regulon (for reviews, see references 12 and 45). The genes constituting the ToxR regulon are encoded both within the ancestral *V. cholerae* genome and on pathogenicity islands derived from the genomes of lysogenic bacteriophages. The *tcp* operon encodes the gene products required for formation of the TCP fiber and is located on the large TCP pathogenicity island also known as the *Vibrio* pathogenicity island (23, 24). The *ctx* operon encodes the CT subunits and is located within the genome of the CTX phage (55). This phage uses TCP as its receptor (55). Both the *tcp* and *ctx* operons are directly activated by ToxT, an AraC homolog that is encoded within the *tcp* operon and which regulates its own expression in addition to that of the other genes (4, 6, 59). Expression of *toxT*, in turn, is dependent on two cytoplasmic membrane protein pairs. The TcpP-TcpH protein pair is encoded by an operon located adjacent to the *tcp* operon on the pathogenicity island, and the ToxR-ToxS protein pair is encoded by an operon located elsewhere on the larger of the two *V. cholerae* chromosomes. Studies on the ToxR-ToxS protein pair have shown that ToxR directly binds DNA and activates transcription (28). Its stability in the membrane is enhanced by ToxS (10, 37). TcpP and TcpH are homologs of ToxR and ToxS, respectively, and are thought to function in a similar manner (18). No additional regulator of the *toxRS* operon is known, and its expression is constitutive over most growth conditions. In contrast, expression of the *tcpPH* operon is responsive to temperature and pH (5) and is dependent on at least two cytoplasmic activators, AphA and the LysR homolog AphB (25, 46). These two activators are encoded by unlinked genes that are not known to be associated with any pathogenicity islands. In addition, the cyclic AMP receptor protein (CRP) represses ToxR regulon gene expression at an early step in the pathway (44). This multitude of regulatory inputs provides a mechanism for virulence gene expression to respond to concurrent signals both within and outside the host.

It is becoming increasingly apparent that expression of many bacterial virulence gene regulons is controlled by overlapping regulatory systems encoded on pathogenicity islands, plasmids, and elsewhere within the genome. A protein that is broadly distributed within members of the family *Enterobacteriaceae* and has been demonstrated to have a role in modulating expression of virulence genes located on plasmids or pathogenicity islands is the histone-like nucleoid structuring protein H-NS. H-NS is a small, abundant protein that was first characterized with respect to its ability to mediate chromosomal DNA condensation (21, 54). H-NS is thought to influence expression of a myriad of seemingly unrelated genes by organizing promoter and regulatory regions into nucleoprotein complexes in response to environmental signals. Expression of genes that are influenced by H-NS is typically responsive to environmental parameters known to influence DNA topology, such as osmolarity, temperature, anaerobiosis, pH, and growth phase (2). H-NS preferentially binds to curved, AT-rich re-

* Corresponding author. Mailing address: Department of Microbiology, HB7550, Dartmouth Medical School, Hanover, NH 03755. Phone: (603) 650-1632. Fax: (603) 650-1318. E-mail: ronald.k.taylor@dartmouth.edu.

gions of DNA and favors the general consensus site 5′-TNT-NAN-3′, where N is any nucleotide (39, 58). Examples of virulence genes best characterized with respect to the influence of H-NS on their expression include the *fim*, *pap*, and *cfa* genes of *Escherichia coli* (13, 17, 22) and several *vir* genes of *Shigella flexneri* (9, 14, 51). In most cases, H-NS modulates virulence gene expression in a negative manner, as evidenced by a large increase in expression under nonpermissive conditions and in the absence of appropriate activator proteins in *hns* mutant strains.

Changes in DNA topology have previously been shown to influence expression of some ToxR regulon genes (35). In this report we investigate the role of *V. cholerae* H-NS on the expression of the ToxR regulon. We have utilized the *V. cholerae* genome sequence to identify a gene encoding a protein with 41% identity to *E. coli* H-NS and have deleted the gene in various virulence gene promoter-*lacZ* fusion strains to determine the influence of H-NS at different levels in the virulence cascade. To further characterize the effect of H-NS at specific promoters, we have deleted genes encoding known activator proteins in various Δ*hns* promoter-*lacZ* fusion strains. Finally, by using promoter deletions, we have determined that H-NS mediates repression over an extensive region upstream of the *toxT* promoter. These results indicate that H-NS influences multiple levels within the *V. cholerae* virulence cascade by repressing gene expression through a mechanism of transcriptional silencing.

MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *V. cholerae* strains were stored at −70°C in Luria-Bertani (LB) medium (27) containing 30% (vol/vol) glycerol. *V. cholerae* was grown in LB broth with a starting pH of either 6.5 or 8.5 at either 30 or 37°C. *E. coli* was grown in LB with a starting pH of 6.5 at 30°C. Antibiotic concentrations used in culture were as follows: ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 15 μg/ml; gentamicin (Gm), 30 μg/ml; and streptomycin (Sm), 100 μg/ml generally or 1 mg/ml when selecting for loss of integrated plasmids from *V. cholerae* following standard allelic exchange. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (XGal) was used in LB agar at 40 μg/ml.

Plasmid and strain construction. Plasmid pMIN1 was constructed by cloning the *Hin*dIII-flanked Gm^r gene from pUCGM (40) into the *Hin*dIII site of pACYC184 (7). This plasmid was used as a counterselection for conjugal matings between *E. coli* and *V. cholerae* recipient strains in this study. The plasmid was cured by overnight growth in LB broth without antibiotics.

The (*toxT-lacZ*)*1* strain MBN032 was constructed by inserting an *Xho*I-*Sal*I fragment containing a promoterless *lacZ* into the unique *Xho*I site within the *toxT* gene of pSAN9 to create pMIN3. Plasmid pSAN9 contains the *V. cholerae* *tcpF*, *toxT*, and *tcpI* genes in pKAS32 (43). The orientation of the *lacZ* fragment was determined by PCR with a primer internal to *lacZ*, and the construct was used for allelic exchange with the O395 Sm^r Δ*lacZ* strain CG842. Proper integration of (*toxT-lacZ*)*1* fusion in MBN032 was confirmed by PCR.

The *V. cholerae* Δ*hns1* mutation was constructed as follows. A sequence from the *V. cholerae* database identified as encoding an H-NS homolog by TBLASTN search (1) provided the basis for the design of oligonucleotide primers MN19 (5′-GATCGATCGCGGCCGCGAAGTTTTCGCCACTTGCCC-3′), MN20 (5′-GATCGATCGCGGCCGCTCTCGCTCAGGAAGACCACG-3′), MN21 (5′-GATCGGAATTCATGGCGCGATTGGCCGCTGC-3′), and MN22 (5′-GATCGTCTAGACCACGCCCTTGAGAAGCGGC-3′), which were used to amplify chromosomal sequences from O395 that flank the *hns* gene. The upstream 1,028-bp and downstream 835-bp fragments were inserted into the allelic exchange vector pKAS32, resulting in the Δ*hns1* plasmid pMIN26. The *hns* deletion was then introduced into the chromosome of various *V. cholerae* strains by allelic exchange. Constructs were verified by PCR.

Construction of the Δ*toxT1* mutation was accomplished by inverse PCR of pSAN9 using primers RT21 (5′-CCCAATCATTGCGTTCTACTCTGAAG-3′) and RT22 (5′-GAATATTTATTTATGTTGACAGGAGTTGCAG-3′). The resultant plasmid, pSAN10, lacks the *toxT* gene. Following allelic exchange with pSAN10, chromosomal deletions were confirmed by PCR.

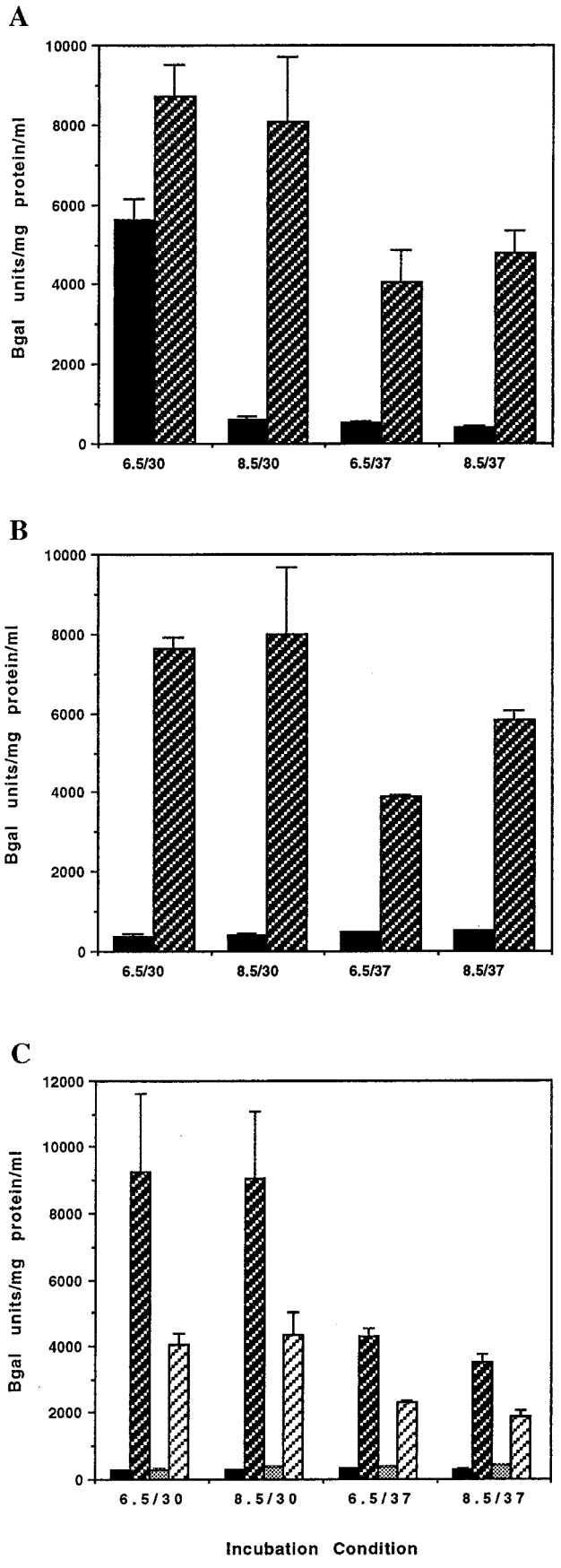
The Δ*tcpP1* mutation was constructed as follows. Two fragments generated by PCR amplification of O395 chromosomal DNA with primers TP-XBA (5′-GATCGTCTAGAAGATTTAGCAAGGTTACCGGG-3′), H-XS (5′-GATCGTCTAGAGAGCTCGAACATTAGGGTAAAGATGAAG-3′), TP-SPH (5′-GATCGGCATGCTTTCCCGATAACCTTTGGTGG-3′) and TP-BAM (5′-GATCGGGATCCAGTGATGCCGGCTAATTCATG-3′) were ligated into the multiple cloning site of pKAS32 to generate the Δ*tcpP1* plasmid pMIN27. Introduction of Δ*tcpP1* into the *V. cholerae* chromosome generates an 11-bp deletion 130 bp from the 3′ end of the gene and inserts 46 bp of noncoding polylinker sequence. The deletion was confirmed by PCR.

The *toxR* mutant strains were constructed via insertional inactivation of the chromosomal copy of *toxR* with pVM55 as previously described (30). All *toxR*::pVM55 strains were maintained in ampicillin. Correct chromosomal insertion of pVM55 was confirmed by observing the OmpU-OmpT switch by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described (30).

The *E. coli* (*toxT-lacZYA*)*4* fusion (long) was constructed by amplifying the *toxT* promoter region from −656 to +77, with respect to the transcriptional start site, using primers RT19 (5′-AAAATCTAGATATGATATTGTGAATGTTGGTGGTG-3′) and RT45 (5′-AAAGGCCTAATCATTGCGTTCTACTCTGAAG-3′). The resulting fragment was cloned into the *lacZ* operon fusion vector

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
E. coli		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* Sm^r	41
DL1976	MC4100 *hns651* Tc^r	57
RT4129	MC4100 (λ*toxT-lacZYA*)*4* (−656/+77)	This work
MBN199	RT4129 *hns651* Tc^r	This work
DH92	TE2680 (Km^r-p*toxT-lacZ*) Km^r	20
RT4146	MC4100 (Km^r-p*toxT-lacZ*) Km^r (−172/+45)	This work
MBN201	RT4146 *hns-651* Tc^r	This work
RT4317	MC4100 (λ*toxT-lacZYA*)*5* (−256/+37)	This work
MBN297	RT4317 *hns651* Tc^r	This work
V. cholerae		
O395 Sm	Classical Ogawa, Sm^r	49
CG842	O395 Sm Δ*lacZ*	8
KSK218	CG842 *ctx-lacZ*	44
MBN032	CG842 (*toxT-lacZ*)*1*	This work
MBN135	CG842 *tcpA-lacZ*	This work
MBN019	KSK218 Δ*toxT1*	This work
MBN142	MBN135 Δ*toxT1*	This work
MBN147	KSK218 Δ*hns1*	This work
MBN148	MBN135 Δ*hns1*	This work
MBN153	MBN019 Δ*hns1*	This work
MBN168	MBN142 Δ*hns1*	This work
MBN170	MBN032 Δ*hns1*	This work
MBN172	MBN032 Δ*tcpP1*	This work
MBN175	MBN172 Δ*hns1*	This work
KSK236	KSK218 *toxR*::pVM55	44
MBN183	MBN170 *toxR*::pVM55	This work
MBN185	MBN019 *toxR*::pVM55	This work
MBN187	MBN175 *toxR*::pVM55	This work
MBN189	MBN032 *toxR*::pVM55	This work
MBN192	MBN153 *toxR*::pVM55	This work
MBN196	MBN147 *toxR*::pVM55	This work
MBN318	MBN172 *toxR*::pVM55	This work
Plasmids		
pKAS32	pGP704 *rpsL* Ap^r	43
pSAN9	pKAS32 *tcpF-tcpJ* Ap^r	This work
pSAN10	pSAN9 Δ*toxT* Ap^r	This work
pMIN1	pACYC184 Gm^r cassette from pUCGM, Gm^r Cm^r	This work
pMIN3	pSAN9 (*toxT-lacZ*)*1* Ap^r	This work
pMIN26	pKAS32, *hns*-flanking sequence, Ap^r	This work
pMIN27	pKAS32, *tcpP*-flanking sequence, Ap^r	This work
pVM55	pJM703.1::*Eco*RI-*Hpa*I ‘*toxR*’ Ap^r	30
pRS415	*lacZYA* transcriptional fusion vector	42
pJYT1	pRS415::(*toxT-lacZYA*)*4*	This work
pMIN38	pRS415::(*toxT-lacZYA*)*5*	This work
pTSK	pACYC184 *toxR*$^+$ *toxS*$^+$ Cm^r	4



pRS415 to generate pJYT1. The (*toxT-lacZYA*)*4* fusion was recombined onto λRS45 (42) and integrated into the chromosome of MC4100 to create RT4129. Similarly, the (*toxT-lacZYA*)*5* fusion (intermediate) was constructed with primers RT52 (5′-AAAAGAATTCAAGTGGTCAAATACTATGTTCTC-3′) and RT53 (5′-AAAAGGATCCGCAGAGAGCCATCCACGTA-3′) to amplify the *toxT* promoter from −256 to +37. This fragment was cloned into pRS415, generating pMIN38. This fusion was also recombined onto λRS45 and lysogenized into MC4100 to yield RT4317. The third *toxT-lacZYA* fusion (short), composed of the region from −172 to +45 of the *toxT* promoter, was previously constructed as a linearized plasmid integrant in the chromosome of *E. coli* strain DH92 (20). This fusion was transduced into MC4100 using P1*vir*, resulting in strain RT4146. The *hns651* mutation from DL1976 was transduced into each of the fusion strains by P1*vir*, and plasmid pTSK was introduced by calcium chloride transformation.

β-Galactosidase assay. β-Galactosidase activity was determined by the method of Miller (27) with the following modifications. In strains of the KSK218 (*ctx-lacZ*) background, cultures were assayed after 16 h of growth. Due to TCP-mediated bacterial autoagglutination of these strains, specific activity was calculated using the protein concentration determined by the bicinchoninic acid procedure (Pierce) rather than the optical density at 600 nm of the culture. For *V. cholerae* strains of the MBN032 background and *E. coli* strains, cultures were assayed during mid-log-phase growth.

SDS-PAGE and immunoblot. Protein extracts from overnight cultures were prepared and analyzed by SDS–12.5% PAGE as described (49). Proteins were transferred to nitrocellulose and probed with anti-TcpA antibody (48) using the Renaissance Chemiluminescence Reagent Plus (NEN Life Science Products).

RESULTS

***V. cholerae* encodes an H-NS homolog.** A TBLASTN search (1) of the *V. cholerae* genome revealed the presence of a gene encoding an open reading frame with 41% identity and 51% similarity to the *E. coli* H-NS protein. The current genome sequence places the beginning of the *hns* gene at position 1221584 of the large chromosome. This gene has also recently been termed *vicH* by Bertin et al. (3). In order to determine any role of *V. cholerae* H-NS in the regulation of virulence gene expression, a deletion of the gene was constructed on a suicide plasmid that could be incorporated into reporter strains by allelic exchange. Introduction of the *hns* deletion into the genome of *ctx-lacZ* fusion strain KSK218 resulted in small, intensely blue colonies on agar containing XGal. The small colony size is consistent with the *hns* phenotype seen in other bacterial species, and the color suggested that a mutation in *hns* might cause an increase in *ctx* gene expression.

Deletion of *hns* results in high levels of *ctx* gene expression. Regulation of expression from the *ctx* operon promoter is thought to be the last step in the ToxR virulence cascade. We therefore chose to first investigate any possible effect of H-NS at this promoter since it responds to many levels of input into the cascade. In *V. cholerae* O1 strains of the classical biotype, *ctx* transcription is optimally induced by growth in vitro at 30°C in LB with a starting pH of 6.5. Expression is reduced under growth conditions of increased temperature and starting pH, with growth at 37°C in LB with a starting pH of 8.5 used as a standard maximal repressing condition.

A comparison of *ctx-lacZ* expression between *hns*$^+$ strain KSK218 and *hns* mutant strain MBN147 grown under various conditions revealed that the Δ*hns1* deletion resulted in derepression of *ctx-lacZ* expression under all growth conditions examined (Fig. 1A). Expression from the *hns* mutant strain at

FIG. 1. β-Galactosidase (Bgal) production in *ctx-lacZ* fusion strains. (A) Δ*hns1* mutation derepresses *ctx* expression under all conditions (pH/°C). Solid bars, KSK218 (*hns*$^+$); hatched bars, MBN147 (Δ*hns1*). (B) Loss of *ctx* expression in the Δ*toxT1* background is restored in the presence of the Δ*hns1* mutation. Solid bars, MBN019 (Δ*toxT1 hns*$^+$); hatched bars, MBN153 (Δ*toxT1* Δ*hns1*). (C) Mutation of both *toxR* and *toxT* results in less *ctx* expression than for either mutation alone in the Δ*hns1* strain. Solid bars, KSK236 (*toxT*$^+$ *hns*$^+$ *toxR*); dark hatched bars, MBN196 (*toxT*$^+$ Δ*hns1 toxR*); shaded bars, MBN185 (Δ*toxT1 hns*$^+$ *toxR*); light hatched bars, MBN192 (Δ*toxT1* Δ*hns1 toxR*). Error bars show standard deviations.

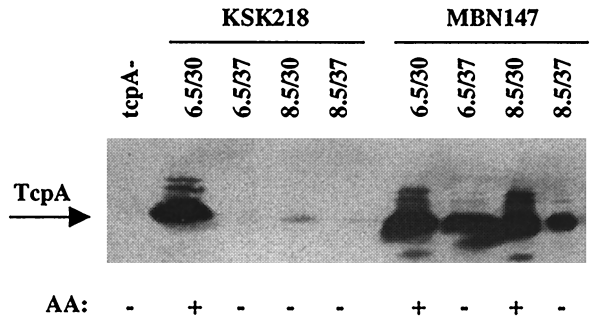


FIG. 2. TcpA production and autoagglutination (AA) by *ctx-lacZ* fusion strain KSK218 and its *hns* derivative MBN147. Strains were cultured under the conditions indicated (pH/°C) for each lane, and 15 μg of total protein extract was subjected to Western blot analysis with anti-TcpA. The *tcpA* mutant control strain was grown at pH 6.5 and 30°C.

30°C, regardless of pH, actually exceeded optimal wild-type expression at 30°C and pH 6.5. At 37°C and either pH, *ctx* expression in the *hns* mutant approached that observed for the *hns*$^+$ strain grown under optimal expression conditions. At 30°C and pH 6.5, the finding that the level of expression in the *hns* mutant exceeded that of the wild type suggests that H-NS exerts a partial repressive effect even under inducing conditions. Together with the finding that loss of H-NS completely overrides the repressive effects of high pH, these results suggest that H-NS plays a role in silencing the *ctx* promoter under various environmental conditions. However, since Δ*hns1* does not permit maximal expression of *ctx-lacZ* at 37°C, this implies that repression by temperature may be influenced by other factors in addition to H-NS.

Expression of *ctx* no longer requires ToxT or ToxR in an *hns* mutant. If H-NS exerts its influence directly at the *ctx* promoter, an *hns* deletion mutant might be derepressed for *ctx* expression even in the absence of the ToxT protein, which activates *ctx* expression by direct interaction at the *ctx* promoter (6). The dependence of *ctx-lacZ* expression on ToxT in LB (pH 6.5) at 30°C is readily apparent by comparing strain KSK218 (Fig. 1A) with the Δ*toxT1* strain MBN019 (Fig. 1B). Strikingly, deletion of *hns* in the Δ*toxT* mutant strain MBN153 was found to restore high levels of *ctx-lacZ* expression under all conditions examined (Fig. 1B). The levels of *ctx-lacZ* expression in the Δ*toxT* Δ*hns* strain MBN153 were found to be essentially identical to those in the *toxT*$^+$ Δ*hns* strain MBN147 (Fig. 1A and B). These results suggest that H-NS mediates repression of gene expression directly at the *ctx* promoter in a manner that can be counteracted by the action of ToxT.

The expression of *ctx* is activated by ToxT, whereas the expression of ToxT is activated by the combined action of the ToxRS and TcpPH membrane protein pairs (18). Since ToxR is required to activate *toxT* expression, the effect of an *hns* mutation on *ctx* expression in a *toxR* mutant should be similar to that shown above for the *toxT* mutant. As shown in Fig. 1C, the normal dependence of *ctx* expression on ToxR, as evidenced in strain KSK236, was relieved by deletion of the *hns* gene in strain MBN196. As expected, the level of *ctx-lacZ* expression in strain MBN196 was found to be similar to that of the *toxT hns* double mutant MBN153 (Fig. 1B). However, an additional contribution of ToxR to *ctx* expression was suggested when the influence of H-NS on *ctx* expression was examined in a *toxR toxT* double mutant. As shown in Fig. 1C, the Δ*toxT toxR*::pVM55 double mutant MBN185 was repressed for *ctx-lacZ* expression under all conditions examined, and transcription was restored upon introduction of the *hns* deletion into the double mutant MBN192. Surprisingly, expression did not increase to the levels achieved with either the Δ*toxT* strain MBN153 (Fig. 1B) or the *toxR*::pVM55 strain MBN196 in the presence of the *hns* allele. If the only contribution of ToxR to *ctx* expression is to activate *toxT* expression, it would be expected that *ctx-lacZ* expression in the Δ*toxT* Δ*hns* strain MBN153 would be identical to that of the Δ*toxT* Δ*hns toxR* strain MBN192. The finding that the double *toxT toxR* mutant cannot achieve the same level of *ctx* expression as either mutation alone in the *hns* background suggests that either ToxT or ToxR can independently activate *ctx* expression in the absence of H-NS. This finding, although not expected in *V. cholerae*, is similar to previous results showing that either ToxR or ToxT can independently activate *ctx* expression in *E. coli* (11, 29).

Expression of *tcpA* is increased in parallel to *ctx* expression in an *hns* mutant. To discern whether promoters within the ToxR virulence cascade in addition to *ctx* might be affected by H-NS, we determined the influence of the *hns* mutation on *tcpA* expression. Protein extracts from overnight cultures of either KSK218 (*hns*$^+$) or MBN147 (Δ*hns1*) grown under the four conditions were subjected to SDS-PAGE and Western immunoblot analysis with anti-TcpA antibody. As expected, significant TcpA production was detected from strain KSK218 only after growth in the optimal inducing conditions of pH 6.5 and 30°C (Fig. 2). However, the presence of the *hns* mutation in strain MBN147 led to TcpA production under all four growth conditions. The trend in TcpA production appeared to essentially parallel the level of *ctx* transcription under the various growth conditions (Fig. 1A). A second measure of TcpA expression is the bacterial autoagglutination that occurs when large amounts of TCP are present on the bacterial surface. Autoagglutination of overnight cultures was evident for the wild-type strain grown at pH 6.5 and 30°C and for the *hns* mutant grown at pH 6.5 and 30°C or pH 8.5 and 30°C. This pattern was consistent with the Western blot analysis, showing that the highest levels of TcpA expression occurred under these conditions. These results indicate that H-NS acts to negatively influence *tcpA* expression, either by acting at the *tcpA* promoter or by influencing prior steps within the regulatory cascade.

In order to determine whether H-NS acts directly at the *tcpA* promoter, we examined the effect of the *hns* mutation on the level of TcpA produced in a *toxT* mutant background. As shown in Fig. 3, the *hns*$^+$ *ctx-lacZ* strain KSK218 produced high levels of TcpA at pH 6.5 and 30°C and less at pH 8.5 and 30°C, whereas no TcpA was detected from the Δ*toxT ctx-lacZ*

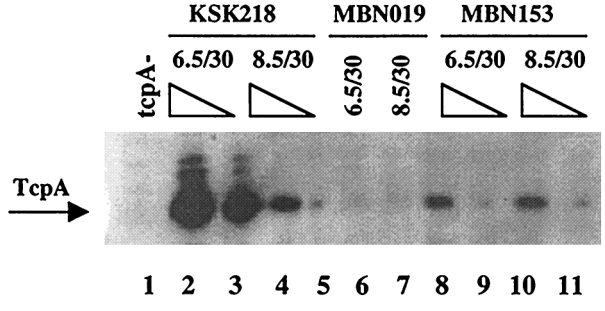
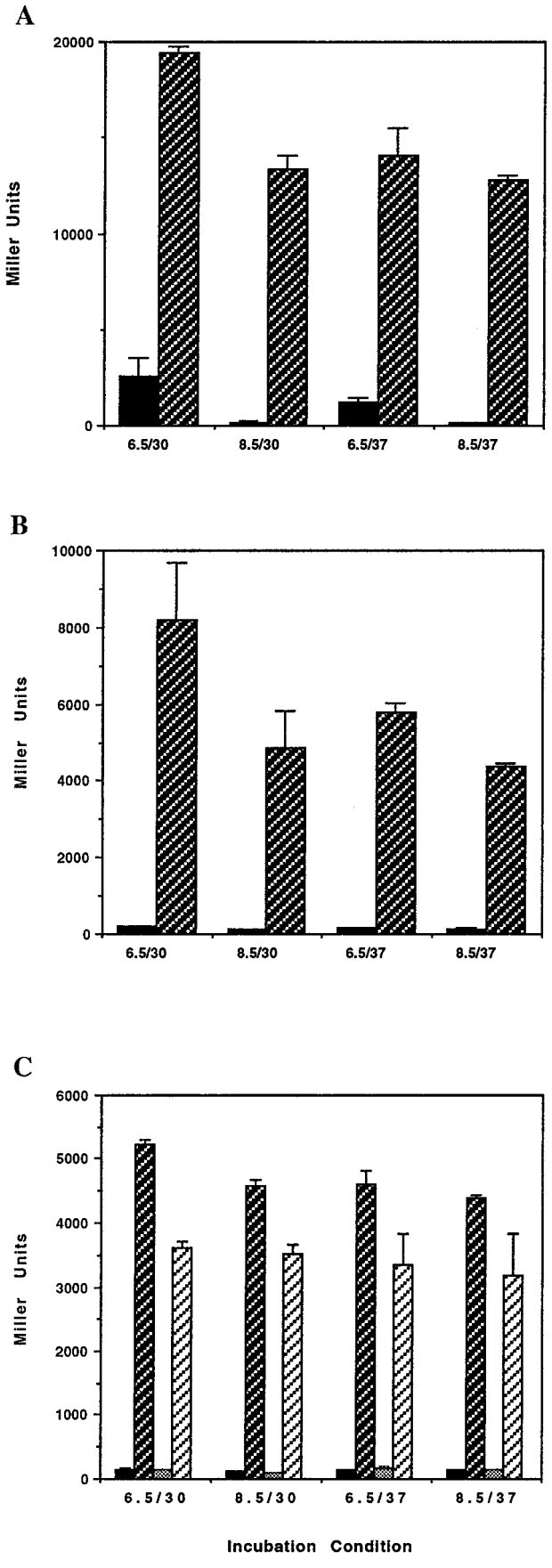


FIG. 3. Western immunoblot with anti-TcpA in *toxT* and *hns* mutant strains. Protein extracts were prepared from strains grown under the indicated conditions (pH/°C) and either 15 μg of total protein extract or a subsequent 1:5 dilution was loaded onto the gel. Lane 1, Δ*tcpA* control at pH 6.5 and 30°C; lanes 2 and 3, KSK218 (*toxT*$^+$ *hns*$^+$); lanes 4 and 5, KSK218; lanes 6 and 7 MBN019 (Δ*toxT1 hns*$^+$); lanes 8 and 9, MBN153 (Δ*toxT1* Δ*hns1*); lanes 10 and 11, MBN153.



strain MBN019 for either growth condition. In contrast, the Δ*toxT* Δ*hns ctx-lacZ* strain MBN153 produced TcpA under both growth conditions, albeit only at a level comparable to that expressed by a wild-type strain grown under the semirepressive condition of LB at pH 8.5 and 30°C. The expression of TcpA in the absence of *toxT* suggests that H-NS directly affects the *tcpA* promoter. However, since TcpA production was not restored to wild-type levels, the effect of H-NS at the *tcpA* promoter appears to be smaller than at the *ctx* promoter, where wild-type levels were achieved. Interestingly, the low level of expression appears to be constitutive with respect to pH (Fig. 3, lanes 8 to 11).

H-NS represses *toxT* expression. The high level of TcpA produced under the nonpermissive condition of pH 8.5 and 30°C in the *toxT*$^+$ Δ*hns* strain MBN147 compared to that of the Δ*toxT* Δ*hns* strain MBN153 (Fig. 2 and 3) suggested that H-NS might directly influence *toxT* expression in addition to its role on expression from the *ctx* and *tcpA* promoters. To investigate this further, we used a series of *toxT-lacZ* fusion strains to directly examine the role of H-NS on *toxT* expression. Introduction of a *toxT-lacZ* transcriptional fusion into a wild-type strain (MBN032) revealed that *toxT* is most highly expressed during the logarithmic stage of growth (data not shown) at pH 6.5 and 30°C. Expression was found to be slightly attenuated at 37°C and greatly reduced by pH 8.5 at either incubation temperature (Fig. 4A). Thus, *toxT* expression responds most significantly to changes in pH but is also affected by temperature. Notably, this regulation mimics that previously determined for *tcpP*, which encodes an activator of *toxT* expression (5, 46).

Incorporation of the *hns* mutation into the *toxT-lacZ* strain MBN170 resulted in significant derepression of expression under all conditions (Fig. 4A). As was observed for *ctx* expression, the most significant derepression occurred during growth under suboptimal expression conditions. At pH 6.5 and 30°C, expression was increased 8-fold in the *hns* mutant, whereas at pH 8.5 and 37°C, there was an 87-fold increase in expression. Interestingly, the trend of slight attenuation by temperature and a greater repressive influence of high pH was still observed. It is noteworthy that an insertion in *hns* was one of several mutations previously reported by Häse and Mekalanos (19) to increase expression of *toxT*.

Similar to the manner in which we examined the role of H-NS at the *ctx* promoter, we determined whether the presence of the activators of *toxT* expression, ToxR and TcpP, were required for derepression in the absence of *hns*. In the *hns*$^+$ *toxR* strain MBN189, the loss of *toxR* abolished transcription of *toxT-lacZ*, as expected (Fig. 4B). However, in the absence of *hns* (strain MBN183), *toxR* was found not to be necessary for *toxT* expression (Fig. 4B). Similar results were found with respect to the requirement of TcpP for *toxT* expression. Deletion of *tcpP* in MBN172 significantly reduced *toxT* expression (Fig. 4C), whereas the level of β-galactosidase in the Δ*tcpP* Δ*hns toxT-lacZ* strain MBN175 was similar to that of the wild type (Fig. 4C). Interestingly, the level of *toxT-lacZ* expression in the *tcpP toxR* double mutant strain in the *hns* mutant background (MBN187) was not as high as in the presence of either activa-

FIG. 4. β-Galactosidase production by *toxT-lacZ* fusion strains. (A) The Δ*hns1* mutation derepresses *toxT* expression under all conditions (pH/°C). Solid bars, MBN032 (*hns*$^+$); hatched bars, MBN170 (Δ*hns1*). (B) The Δ*hns1* mutation permits *toxT* expression in a strain that lacks ToxR. Solid bars, MBN189 (*hns*$^+$ *toxR*); hatched bars, MBN183 (Δ*hns1 toxR*). (C) Neither ToxR nor TcpP is required to activate *toxT* in the absence of *hns*. Solid bars, MBN172 (*hns*$^+$ Δ*tcpP1*); dark hatched bars, MBN175 (Δ*hns1* Δ*tcpP1*); shaded bars, MBN318 (*hns*$^+$ Δ*tcpP1 toxR*); light hatched bars, MBN187 (Δ*hns1* Δ*tcpP1 toxR*). Error bars show standard deviations.

A)

Fusion Strain	*hns*$^+$	*hns*$^+$/ToxR$^+$	*hns*$^-$	*hns*$^-$/ToxR$^+$
RT4146 (short)	214±43	42±1	334±66	84±16
RT4317 (intermediate)	46±1	83±0	435±2	255±40
RT4129 (long)	20±3	60±4	346±39	272±3

B)

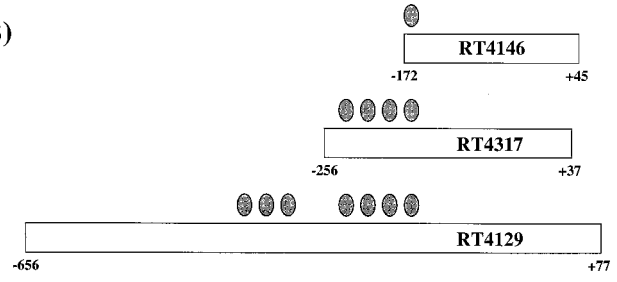


FIG. 5. Differential responses of *toxT-lac* fusions containing various amounts of the promoter region to H-NS and ToxR. (A) β-Galactosidase activity (Miller units, means ± standard deviations) from *E. coli* strains that are either *hns*$^+$ or *hns651* and carry a chromosomal copy of each fusion construct. ToxR is supplied from plasmid pTSK. (B) Schematic representation of the extent of the *toxT* promoter present in each fusion construct. Each oval represents an approximately twofold relative influence contributed by H-NS to decrease the basal level of β-galactosidase activity.

tor mutation alone (Fig. 4B and C). As in the case of the *ctx* promoter, this suggests that in the absence of H-NS, each activator can contribute independently to increase *toxT* expression. Of further interest was the trend of the *toxR* mutants to retain the characteristic regulatory pattern of *toxT* expression conferred by pH and temperature (Fig. 4B), whereas this fluctuation was absent in the *tcpP* mutant backgrounds (Fig. 4C). This is consistent with the nearly constitutive expression of ToxR for all of the growth conditions used in this assay (34) versus the regulated expression of TcpP under these conditions (5).

H-NS functions at a region upstream of the *toxT* promoter. It has previously been shown that expression of a *toxT-lacZ* transcriptional fusion encompassing the region from −172 to +45 of the ToxR-dependent *toxT* transcriptional start site has a high basal level of activity that is actually repressed rather than activated by ToxR expressed in *E. coli* (20). The findings described above showing that H-NS exerts a repressive activity on *toxT* expression suggested that the high-level constitutive expression observed for this fusion construct might be due to deletion of sequences required for H-NS interaction near the *toxT* promoter. To examine this possibility, we constructed a series of *toxT* fusions with various lengths of upstream DNA and determined their expression in various *toxR* and *hns* backgrounds (Fig. 5). These included the original fusion transduced into the chromosome of strain MC4100 (short fusion strain RT4146), as well as two additional transcriptional fusions containing more extensive upstream regions spanning positions −256 to +37 (intermediate fusion strain RT4317) or −656 to +77 (long fusion strain RT4129). The latter two fusions were constructed as lambda lysogens of MC4100. These two fusions yielded substantially lower units of basal activity in the absence of ToxR than the original short fusion and, unlike the short fusion, were activated by ToxR (Fig. 5A).

To determine whether H-NS contributed to the reduced basal level of expression from the fusions containing more extensive upstream regions, the *hns651* mutation was transduced into the fusion backgrounds, and the effect on transcription was assessed by β-galactosidase assay. The *hns651* mutation was found to elevate the level of transcription of these fusions to an approximately equivalent level at pH 6.5 and 30°C (Fig. 5A). The shortest fusion showed a 1.6-fold increase in basal expression in the *hns* strain, whereas the intermediate and long fusions showed 9-fold and 14-fold increases, respectively. These results suggest that the fusions with the more extensive regions upstream of the promoter are more strongly repressed by H-NS. Interestingly, although the intermediate and long fusions were moderately activated by ToxR in an *hns*$^+$ background, they were repressed by ToxR in an *hns* mutant background. The repression of *toxT* expression by ToxR was similar to that seen for the short fusion regardless of *hns* background. This suggests a role for H-NS in the normal regulation of *toxT* expression and that this regulation is lacking in the short fusion strain. A model accounting for the influence of H-NS on the *toxT* promoter regions from each of the three fusion lengths is shown in Fig. 5B. Each oval represents an approximately twofold repression by H-NS on *toxT* expression. These data are consistent with a mechanism by which H-NS represses transcription by interactions at regions located at significant distances upstream of the RNA polymerase binding site. This type of repression by H-NS has been termed transcriptional silencing (17).

DISCUSSION

Regulation of the expression of the genes that encode the major virulence determinants of *V. cholerae*, CT and TCP, involves a complex interplay between regulators encoded within the ancestral genome and those encoded within the

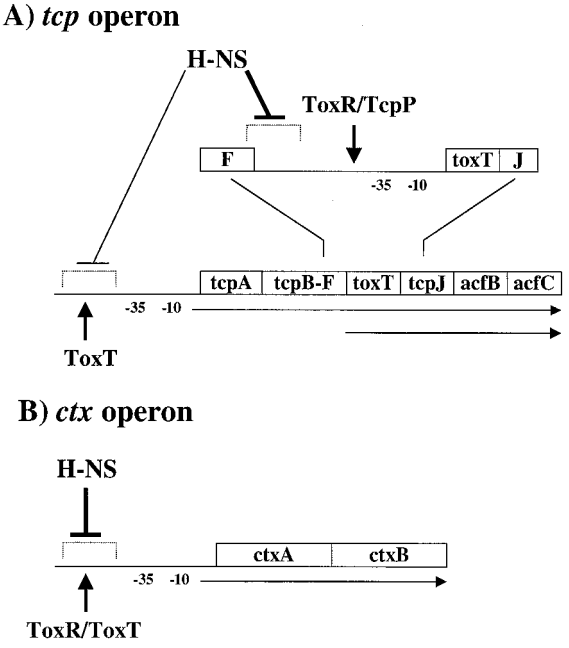


FIG. 6. Model for H-NS-mediated negative modulation of virulence gene expression. (A) H-NS negatively affects two steps in *tcp* operon expression. A major effect occurs at the ToxR/TcpP-dependent transcriptional start site upstream of *toxT*. The major region required for this activity is located upstream of position −172 with respect to the *toxT* mRNA start site. The negative effect of H-NS at the *toxT* promoter is overcome by ToxR and TcpP. H-NS also exerts a minor effect at the *tcp* operon promoter located upstream of *tcpA*. This effect is overcome by ToxT. (B) H-NS also exerts a major negative effect at the *ctx* promoter. This effect is counteracted by ToxT, with a possible additional contribution from ToxR.

TCP pathogenicity island. The regulators encoded within the TCP island include the AraC homolog ToxT, which activates the expression of the *ctx* and *tcp* genes, and the transmembrane protein pair TcpP-TcpH, which activate the expression of *toxT*. Other virulence gene regulators that are not exclusively associated with pathogenicity islands and that likely participate in additional regulatory networks within the cell include the transmembrane protein pair ToxR-ToxS and the cytoplasmic proteins AphA and AphB. ToxR-ToxS is present in many *Vibrio* species and, in addition to its role in *toxT* activation, is required for expression of genes encoding homologs of the *V. cholerae* OmpU protein (56). AphA and AphB are required for expression of the *tcpPH* operon (25, 46), but other potential roles for these proteins have not yet been elucidated. In addition, gene expression within the *V. cholerae* ToxR virulence regulon is negatively influenced by the cyclic AMP-cyclic AMP receptor protein complex (44). The mechanism for this negative regulation within the virulence cascade is still under investigation, but it is known that this regulatory system influences the expression of multiple genes that affect cellular physiology in response to carbon source and perhaps additional growth parameters. In the present study we have determined that another protein with global effects, the H-NS protein, which influences chromatin structure and gene expression in response to numerous growth parameters, has a major negative influence at multiple levels of expression within the ToxR virulence gene regulon.

As depicted in Fig. 6, the studies reported here indicate that H-NS influences ToxR regulon gene expression by exerting a negative effect on at least three promoters, *toxT*, *tcpA*, and *ctx*. In the absence of H-NS, expression from each of these promoters was increased dramatically under noninducing conditions and, to a smaller degree, under inducing conditions. These results suggest that H-NS plays a role in repressing ToxR regulon gene expression under environmental conditions not normally permissive for its expression and that it represses expression even under normally inducing conditions. The observation that an *hns* mutation derepresses the expression of *toxT*, *ctx*, and *tcpA* under several environmental conditions even in the absence of their cognate activator proteins suggests that H-NS is directly influencing these promoters.

H-NS appears to exert its largest repressive effect on the *toxT* promoter. Expression of *toxT* is activated by ToxR-ToxS together with TcpP-TcpH (18). In the absence of H-NS, expression of *toxT* was close to or greater than wild-type levels under inducing conditions in the absence of either ToxR, TcpP, or both. This was the case even under normally repressive environmental conditions. These findings are further supported by the recent report of an insertion mutation in *hns* that significantly increased *toxT* transcription (19). Analysis of *E. coli toxT-lacZ* fusions suggests that H-NS exerts its negative effects on transcription by influencing the promoter over an extensive region from −172 to beyond −256 with respect to the start of transcription (Fig. 5 and 6). The region of the promoter spanning from −114 to −73 has been shown to be required for the interaction of ToxR with DNA (20). The location of the TcpP binding site has not yet been reported but TcpP appears to influence transcription downstream of −172 (31). Previous experiments with *toxT-lacZ* fusions carrying DNA only to −172 (short) have indicated that *toxT* is actually repressed by ToxR (20). This paradoxical finding may be explained by the results shown here, that upon increasing the length of the upstream region in these fusions, to −256 or to −656, the basal level of expression is decreased by H-NS such that it becomes activated by ToxR.

H-NS also has a significant effect on expression of the *ctx* promoter. This promoter is directly activated by ToxT in *V. cholerae* (6). Although at least some influence on the *ctx* promoter in the *toxT*$^+$ background may be the result of increased expression of the *toxT* promoter, as discussed above, deletion of *hns* resulted in expression from *ctx* that was close to or greater than wild-type expression in the absence of ToxT. This result suggests that H-NS also influences the *ctx* promoter directly. Interestingly, the level of *ctx* expression in the *hns* mutant lacking both ToxT and ToxR is lower than the level of expression in an *hns* mutant lacking only ToxT. This result suggests that ToxR directly influences the *ctx* promoter in the absence of *hns*. It has previously been shown in *E. coli* that either ToxR or ToxT is capable of activating *ctx* expression (11, 28). Genetic footprint analysis indicates that ToxR interacts at two positions, one at −69 to −57 and the other at −47 to −39 (36). Although a direct role for ToxR at the *ctx* promoter in *V. cholerae* has not been demonstrated in vitro, recent studies indicate that ToxR and ToxT have a dual role at the *ctx* promoter in vivo (26).

H-NS has a more moderate effect at the *tcpA* promoter than at the *toxT* and *ctx* promoters. In a *toxT* mutant background, the absence of H-NS restored expression to wild-type levels at pH 8.5 and 30°C but not at pH 6.5 and 30°C. ToxT is the only known activator that functions at the *tcpA* promoter, but the cyclic AMP-cyclic AMP receptor protein complex has been implicated in exerting a negative influence at the *tcpA* promoter. Further investigation of how these factors interact to influence expression from the *tcpA* promoter is under way.

The results presented here indicate that in *V. cholerae*, H-NS affects both the expression of a positive transcriptional regulator, ToxT, and the expression of the target genes of the regulator, *tcpA* and *ctx*. This is similar to the situation observed in the VirF-VirB regulatory cascade of *S. flexneri* (38). The *toxT*, *tcpA*, and *ctx* promoters possess characteristics that have been correlated with H-NS binding. The high AT content of these promoters likely promotes local curvature within these regions. Molecular models for transcriptional silencing by H-NS vary with respect to the position at which the protein interacts with the DNA. For genes in which the H-NS binding sites overlap the promoter elements directly, H-NS is proposed to reduce transcription by preventing the binding of RNA polymerase at the promoter (53). Repression of *E. coli rrnB* (50) and *S. flexneri virB* (51) is thought to occur in this manner. Alternatively, many H-NS binding sites have been found to lie outside the immediate promoter region. This appears to be the situation with *toxT*. In the case of *Salmonella enterica* serovar Typhimurium *proU* expression, the H-NS binding site is a curved region 200 bp downstream from the transcriptional start site (33). For *proU* and other genes where H-NS binds outside of the promoter elements, H-NS-DNA binding causes a change in DNA topology that leads to a subsequent influence on gene expression (33). In such a case, H-NS binding could generate locally constrained supercoiling that specifically silences the promoter (52). Finally, it has been suggested that H-NS can repress transcription by decreasing the rate of open complex formation at the promoter (47).

The mechanisms by which activator proteins function to counteract H-NS-mediated modulation of gene expression are not well understood. Atlung and Ingmer (2) have suggested that H-NS generally functions as an activator antagonist at genes for which expression is repressed by H-NS and specifically induced by positive regulators. Examples include the *cfaAB*, *pap*, and *coo* genes of *E. coli* that are repressed by H-NS and activated by CfaD, PapB, and Rns, respectively (15, 22, 32). Although these activators are dispensable in the absence of H-NS, CfaD and Rns are able to further increase transcrip-

tion of their target genes in *hns* mutant strains (22, 32). A similar situation occurs at the *toxT* and *ctx* promoters. At least one of the functions of such activators appears to be to counteract H-NS repression of their respective target genes. Whether these activators interact directly with H-NS to displace it from the DNA is not known. Other transcriptional activators that function at H-NS-repressed promoters are still required to activate gene expression in the absence of H-NS. For example, expression of the *Shigella virB* gene is repressed by H-NS but still requires VirF for activation in *hns* mutants (51). This appears to be the case for the *tcpA* promoter, since ToxT is still required for maximal expression in the absence of H-NS. Further analysis of the molecular interactions between H-NS and the regulatory proteins TcpP, ToxR, and ToxT at the various promoters within the ToxR regulon will provide insights into the mechanisms by which this protein influences virulence gene expression.

ACKNOWLEDGMENTS

We thank Cori Sandoe and Jennifer Thibert for plasmids, Christine White-Ziegler and Victor DiRita for strains, and Tom Kirn for assistance with genome analysis.

This work was supported by NIH grants AI39654 to R.K.T. and AI41558 to K.S. J.D.P. was supported by postdoctoral fellowship PF-4286 from the ACS.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Atlung, T., and H. Ingmer.** 1997. H-NS: a modulator of environmentally regulated gene expression. Mol. Microbiol. **24:**7–17.
3. **Bertin, P., N. Benhabiles, E. Krin, C. Laurent-Winter, C. Tendeng, E. Turlin, A. Thomas, A. Danchin, and R. Brasseur.** 1999. The structural and functional organization of H-NS-like proteins is evolutionarily conserved in gram-negative bacteria. Mol. Microbiol. **31:**319–329.
4. **Brown, R. C., and R. K. Taylor.** 1995. Organization of *tcp*, *acf*, and *toxT* genes within a ToxT-dependent operon. Mol. Microbiol. **16:**425–439.
5. **Carroll, P. A., K. T. Tashima, M. B. Rogers, V. J. DiRita, and S. B. Calderwood.** 1997. Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae*. Mol. Microbiol. **25:**1099–1111.
6. **Champion, G. A., M. N. Neely, M. A. Brennan, and V. J. DiRita.** 1997. A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains. Mol. Microbiol. **23:**323–331.
7. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
8. **Chiang, S. L., R. K. Taylor, M. Koomey, and J. J. Mekalanos.** 1995. Single amino acid substitutions in the N-terminus of *Vibrio cholerae* TcpA affect colonization, autoagglutination, and serum resistance. Mol. Microbiol. **17:**1133–1142.
9. **Colonna, B., M. Casalino, P. A. Fradiani, C. Zagaglia, S. Naitza, L. Leoni, G. Prosseda, A. Coppo, P. Ghelardini, and M. Nicoletti.** 1995. H-NS regulation of virulence gene expression in enteroinvasive *Escherichia coli* harboring the virulence plasmid integrated into the host chromosome. J. Bacteriol. **177:**4703–4712.
10. **DiRita, V. J., and J. J. Mekalanos.** 1991. Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell **64:**29–37.
11. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**5403–5407.
12. **DiRita, V. J.** 1995. Three-component regulatory system controlling virulence in *Vibrio cholerae*, p. 351–365. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. American Society for Microbiology, Washington, D.C.
13. **Donato, G. M., M. J. Lelivelt, and T. H. Kawula.** 1997. Promoter-specific repression of *fimB* expression by the *Escherichia coli* nucleoid-associated protein H-NS. J. Bacteriol. **179:**6618–6625.
14. **Dorman, C. J., and M. E. Porter.** 1998. The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. Mol. Microbiol. **29:**677–684.
15. **Forsman, K., B. Sonden, M. Goransson, and B. E. Uhlin.** 1992. Antirepression function in *Escherichia coli* for the cAMP-cAMP receptor protein transcriptional activator. Proc. Natl. Acad. Sci. USA **89:**9880–9884.
16. **Gardel, C. L., and J. J. Mekalanos.** 1994. Regulation of cholera toxin by temperature, pH and osmolarity. Methods Enzymol. **235:**517–526.
17. **Göransson, M., B. Sondén, P. Nilsson, B. Dagberg, K. Forsman, K. Emanuelsson, and B. E. Uhlin.** 1990. Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. Nature **344:**682–685.
18. **Häse, C. C., and J. J. Mekalanos.** 1998. TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **95:**730–734.
19. **Häse, C. C., and J. J. Mekalanos.** 1999. Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **96:**3183–3187.
20. **Higgins, D. E., and V. J. DiRita.** 1994. Transcriptional control of *toxT*, a regulatory gene in the ToxR regulon of *Vibrio cholerae*. Mol. Microbiol. **14:**17–29.
21. **Hulton, C. S. J., A. Seirafi, J. C. D. Hinton, J. M. Sidebotham, L. Waddell, G. D. Pavitt, T. Owen-Hughes, A. Spassky, H. Buc, and C. F. Higgins.** 1990. Histone-like protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. Cell **63:**631–642.
22. **Jordi, B. J. A. M., B. Dagberg, L. A. M. de Haan, A. M. Hamers, B. A. M. van der Zeijst, W. Gaastra, and B. E. Uhlin.** 1992. The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/I fimbrial operon of *Escherichia coli*. EMBO J. **11:**2627–2632.
23. **Karaolis, D. K. R., J. A. Johnson, C. C. Bailey, E. C. Boedeker, J. B. Kaper, and P. R. Reeves.** 1998. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc. Natl. Acad. Sci. USA **95:**3134–3139.
24. **Karaolis, D. K. R., S. Somara, D. R. Maneval, Jr., J. A. Johnson, and J. B. Kaper.** 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature **399:**375–379.
25. **Kovacikova, G., and K. Skorupski.** 1999. A *Vibrio cholerae* LysR homolog, AphB, cooperates with AphA at the *tcpPH* promoter to activate expression of the ToxR virulence cascade. J. Bacteriol. **181:**4250–4256.
26. **Lee, S. H., D. L. Hava, M. K. Waldor, and A. Camilli.** 1999. Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. Cell **99:**625–634.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48:**271–279.
29. **Miller, V. L., and J. J. Mekalanos.** 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. Proc. Natl. Acad. Sci. USA **81:**3471–3475.
30. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
31. **Murley, Y. M., P. A. Carroll, K. Skorupski, R. K. Taylor, and S. B. Calderwood.** 1999. Differential transcription of the *tcpPH* operon confers biotype-specific control of the *Vibrio cholerae* ToxR virulence regulon. Infect. Immun. **67:**5117–5123.
32. **Murphree, D., B. Froehlich, and J. R. Scott.** 1997. Transcriptional control of genes encoding CS1 pili: negative regulation by a silencer and positive regulation by Rns. J. Bacteriol. **179:**5736–5743.
33. **Owen-Hughes, T. A., G. D. Pavitt, D. S. Santos, J. M. Sidebotham, C. S. J. Hulton, J. C. D. Hinton, and C. F. Higgins.** 1992. The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell **71:**255–265.
34. **Parsot, C., and J. J. Mekalanos.** 1990. Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat shock response. Proc. Natl. Acad. Sci. USA **87:**9898–9902.
35. **Parsot, C., and J. J. Mekalanos.** 1992. Structural analysis of the *acfA* and *acfD* genes of *Vibrio cholerae*: effects of DNA topology and transcriptional activators on expression. J. Bacteriol. **174:**5211–5218.
36. **Pfau, J. D., and R. K. Taylor.** 1996. Genetic footprint of the ToxR-binding site in the promoter for cholera toxin. Mol. Microbiol. **20:**213–222.
37. **Pfau, J. D., and R. K. Taylor.** 1998. Mutations in *toxR* and *toxS* that separate transcriptional activation from DNA binding at the cholera toxin gene promoter. J. Bacteriol. **180:**4724–4733.
38. **Prosseda, G., P. A. Fradiani, M. Di Lorenzo, M. Falconi, G. Micheli, M. Casalino, M. Nicoletti, and B. Colonna.** 1998. A role for H-NS in the regulation of the *virF* gene of *Shigella* and enteroinvasive *Escherichia coli*. Res. Microbiol. **149:**15–25.
39. **Rimsky, S., and A. Spassky.** 1990. Sequence determinants for H1 binding on *Escherichia coli lac* and *gal* promoters. Biochemistry **29:**3765–3771.
40. **Schweizer, H. P.** 1993. Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–834.
41. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
43. **Skorupski, K., and R. K. Taylor.** 1996. Positive selection vectors for allelic exchange. Gene **169:**47–52.

44. **Skorupski, K., and R. K. Taylor.** 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **94:**265–270.
45. **Skorupski, K., and R. K. Taylor.** 1997. Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol. Microbiol. **25:** 1003–1009.
46. **Skorupski, K., and R. K. Taylor.** 1999. A new level in the *Vibrio cholerae* ToxR virulence cascade: AphA is required for transcriptional activation of the *tcpPH* operon. Mol. Microbiol. **31:**763–771.
47. **Spassky, A., S. Rimsky, H. Garreau, and H. Buc.** 1984. H1a, an *E. coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. Nucleic Acids Res. **12:**5321–5340.
48. **Sun, D. J., M. Sayer, I. Kovari, R. A. Sumrada, and R. K. Taylor.** 1991. Localization of protective epitopes within the pilin subunit of the *Vibrio cholerae* toxin-coregulated pilus. Infect. Immun. **59:**114–118.
49. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
50. **Tippner, D., H. Afflerbach, C. Bradaczek, and R. Wagner.** 1994. Evidence for a regulatory function of the histone-like *Escherichia coli* protein H-NS in ribosomal RNA synthesis. Mol. Microbiol. **11:**589–604.
51. **Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa.** 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by VirF and repression by H-NS. J. Bacteriol. **175:**6142–6149.
52. **Tupper, A. E., T. A. Owen-Hughes, D. W. Ussery, D. S. Santos, D. J. P. Ferguson, J. M. Sidebotham, J. C. D. Hinton, and C. F. Higgins.** 1994. The chromatin-associated protein H-NS alters DNA topology *in vitro*. EMBO J. **13:**258–268.
53. **Ueguchi, C., and T. Minuzo.** 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12:**1039–1046.
54. **Varshavsky, A. J., S. A. Nedospasov, V. V. Bakayev, T. G. Bakayeva, and G. P. Georgiev.** 1977. Histone-like proteins in the purified *Escherichia coli* deoxyribonucleoprotein. Nucleic Acids Res. **4:**2725–2745.
55. **Waldor, M. K., and J. J. Mekalanos.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **272:**1910–1914.
56. **Welch, T. J., and D. H. Bartlett.** 1998. Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9. Mol. Microbiol. **27:**977–985.
57. **White-Ziegler, C. A., M. L. A. Hill, B. A. Braaten, M. W. van der Woude, and D. A. Low.** 1998. Thermoregulation of *Escherichia coli pap* transcription: H-NS is a temperature-dependent DNA methylation blocking factor. Mol. Microbiol. **28:**1121–1137.
58. **Yamada, H., S. Muramatsu, and T. Mizuno.** 1990. An *Escherichia coli* protein that preferentially binds to sharply curved DNA. J. Biochem. **108:**420–425.
59. **Yu, R. R., and V. J. DiRita.** 1999. Analysis of an autoregulatory loop controlling ToxT, cholera toxin, and toxin-coregulated pilus production in *Vibrio cholerae*. J. Bacteriol. **181:**2584–2592.