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Mechanism of ToxT-Dependent Transcriptional Activation at the *Vibrio cholerae tcpA* Promoter†

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The AraC homolog ToxT coordinately regulates virulence gene expression in *Vibrio cholerae*. ToxT is required for transcriptional activation of the genes encoding cholera toxin and the toxin coregulated pilus, among others. In this work we focused on the interaction of ToxT with the *tcpA* promoter and investigated the mechanism of ToxT-dependent transcriptional activation at *tcpA*. Deletion analysis showed that a region from −95 to +2 was sufficient for ToxT binding and activation, both of which were simultaneously lost when the deletion was extended to −63. A collection of point mutations generated by error-prone PCR revealed two small regions required for ToxT-dependent transactivation. Binding studies performed with representative mutations showed that the two regions define sites at which ToxT binds to the *tcpA* promoter region, most likely as a dimer. Results obtained by using a *rpoA* truncation mutation showed that ToxT-dependent activation at *tcpA* involves the C-terminal domain of the RNA polymerase alpha subunit. A model of ToxT-dependent transcriptional activation at *tcpA* is proposed, in which ToxT interacts with two A-rich regions of *tcpA* centered at −72 and −51 and requires the alpha C-terminal domain of RNA polymerase.

Vibrio cholerae is the etiological agent of the life-threatening diarrheal disease cholera. This bacterium inhabits an aquatic environmental niche when it is not infecting a human host and is acquired through ingestion of contaminated food or drinking water. *V. cholerae* colonizes the upper intestine of humans via the toxin coregulated pilus (TCP) (25, 63), one of the organism's two main virulence factors. The second major virulence factor is cholera toxin (CT), which is responsible for the efflux of fluids into the intestine that results in diarrhea (33). Expression of both TCP and CT is regulated by various environmental factors, such as temperature, pH, osmolarity, bile, CO_2, and amino acids (reviewed in references 9 and 60). This regulation by external stimuli ensures that TCP and toxin are expressed only when they are needed (i.e., in the host). The genes required for TCP biogenesis are located on the *Vibrio* pathogenicity island (15, 34). The CT subunits are encoded on a separate genetic element, the lysogenic CTXϕ bacteriophage (68). These two elements are thought to have been acquired separately through horizontal gene transfer, and it has been shown that CTXϕ uses TCP as its receptor (68).

Regulation of genes encoding TCP and CT is controlled at the level of transcription by a regulatory cascade referred to as the ToxR virulence regulon (9, 12, 60). ToxR is a transmembrane protein with homology to the OmpR family of DNA binding proteins (44) that, along with its partner ToxS, is encoded within the ancestral *V. cholerae* genome. ToxR directly binds to DNA (44), while ToxS enhances the ability of ToxR to form a transcriptionally active complex (11, 41, 52). The *toxRS* operon is expressed constitutively under most growth conditions. ToxR and ToxS have other roles in *V. cholerae* besides virulence gene regulation (10, 43). One example is regulation of the relative abundance of the porins OmpU and OmpT. Transcription of *ompU* is positively regulated by ToxR, while *ompT* is repressed by ToxR. This ToxR-dependent porin modulation has recently been shown to have an important role in pathogenesis (54). When *ompT* is expressed in a $ToxR^+$ strain in place of *ompU*, the levels of *ctx* and *tcp* transcription decrease and colonization is reduced. It has been proposed that the importance of porin regulation led to the recruitment of ToxR as a regulator of the acquired pathogenicity island virulence genes, such as *tcp* and *ctx* (54).

A second pair of membrane-bound proteins, TcpP and TcpH, have homology to ToxR and ToxS, respectively, and are thought to function in a similar manner (22). TcpP and TcpH are encoded on the TCP-ACF pathogenicity island, and their expression is influenced by factors such as temperature and pH in a manner that parallels what happens in *tcp* and *ctx* gene expression (6). Transcription of the *tcpPH* operon is dependent on two regulators which are encoded on the ancestral genome, AphA and the LysR homolog AphB (35, 62).

ToxRS and TcpPH function cooperatively to activate expression of another TCP-ACF-encoded activator, ToxT (12, 22). ToxT is required for activation of the *tcp* and *ctx* operons (3, 7), as well as other genes in the virulence regulon, such as *tcpI*, *acfA*, *tagA*, and *tagD* (12; Taylor, unpublished data). ToxT also regulates its own expression through an autoregulatory loop (3, 70). ToxT therefore functions as a coordinate regulator of virulence gene expression in *V. cholerae* by activating genes encoded on both the TCP-ACF element and the CTX element in response to environmental cues. In addition to the series of activators involved in this regulatory cascade, the global regulators cyclic AMP receptor protein and H-NS have a negative influence on virulence gene expression in *V. cholerae* (49, 61). Both of these regulators influence expression of a number of

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† For a commentary on this article, see p. 5529 in this issue.

genes in the regulon. H-NS has been shown to function directly at the *ctx*, *toxT*, and *tcpA* promoters (49). ToxT overrides the negative effects of these regulators directly at the *tcpA* and *ctx* promoters (49).

ToxT is a member of the AraC/XylS family of transcriptional regulators (26, 50), a family made up almost exclusively of activator proteins. AraC/XylS family members are characterized by a conserved stretch of amino acids at the C terminus, which constitutes the DNA binding domain (reviewed in references 18 and 38). This region of homology contains two helix-turn-helix (HTH) motifs, and crystal structures of AraC family members have shown that these HTH motifs interact with the DNA helix in the major groove (36, 55). Many AraC family members have binding sites that are adjacent to or overlap the −35 region, and interactions between AraC family members and RNA polymerase have been suggested (1, 18, 27, 28, 30, 31, 38). The N terminus of AraC family members is not conserved and has been shown for some family members to be involved in dimerization and binding of small effector molecules (18, 38).

Proteins in the AraC/XylS family fall into three main groups depending on the types of genes that they regulate. Family members that regulate carbon metabolism, such as AraC of *Escherichia coli*, are known to respond to small effector molecules that bind to the N-terminal domain of the protein. In the case of AraC, the effector molecule is arabinose. The members of a second group of the AraC family regulate genes involved in stress response. These proteins include SoxS, Rob, and MarA, and they have been shown to function as monomers (38). A third group, including ToxT, Rns of enterotoxigenic *E. coli* (45), BfpT (PerA) of enteropathogenic *E. coli* (66), and ExsA of *Pseudomonas aeruginosa* (29), regulate virulence gene expression. These virulence regulators may respond to physical cues, such as temperature and pH; only one virulence regulator of the AraC/XylS family, UreR, has been shown to directly respond to an effector molecule, urea (65). Recently, the effect of bile on regulation of virulence genes in *V. cholerae* has been investigated (20, 57). The addition of bile to growth media repressed transcription of *tcp* and *ctx* but, paradoxically, induced transcription of *toxT*. This suggests that bile may directly influence the transcriptional activity of the ToxT protein itself, perhaps by binding to the N-terminal domain (57).

Several promoters in *V. cholerae* that are regulated by ToxT have been characterized, and there is no obvious consensus sequence that can be proposed as the ToxT binding site. Many AraC/XylS family members recognize asymmetric sequences (18, 45), which makes it difficult to predict the binding sites of these proteins. We have focused most of our work in this regard on defining the *cis*-active requirements for ToxT interaction at the *tcp* operon promoter and investigating the mechanism of ToxT-dependent transcriptional activation at *tcpA*. Using a promoter deletion analysis along with PCR-based mutagenesis of the promoter region, we found that ToxT interacts with two A-rich regions centered at −72 and −51. The mutations that prevent ToxT-mediated activation of *tcpA* expression are clustered within these two regions, which we have designated site I (−72) and site II (−51). Using gel shift analysis, we determined that a His-tagged version of ToxT binds to a probe that includes both site I and site II. ToxT does not detectably bind to either site I or site II alone. ToxT also does not detectably bind to probes carrying various point mutations in site I, but it does bind weakly to a probe carrying a point mutation in site II. The region proposed for ToxT binding at the *tcp* operon promoter does not contain any obvious dyad symmetrical sequences. Upon binding to these sites ToxT appears to activate transcription of the *tcp* operon via a mechanism that involves the alpha C-terminal domain (αCTD) of RNA polymerase.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics. The strains and plasmids used in this study are listed in Table 1. *V. cholerae* and *E. coli* strains were maintained at −70°C in Luria-Burtani (LB) medium (40) containing 30% (vol/vol) glycerol. *E. coli* was grown in LB medium with a starting pH of either 7.0 or 6.5 at 37 or 30°C. *V. cholerae* was grown at 30°C in LB medium with a starting pH of 6.5. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml; and streptomycin, 100 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to LB agar at a concentration of 40 μg/ml. Arabinose was added to the growth media at a final concentration of 0.02%.

Plasmid and strain construction. The *tcpA-lacZ* deletion series in pRS415 was constructed as follows. The *tcpA* promoter fragments for pAJA3, pRH82, pRH72, pAJA2, pAJA1, and pRH8 were amplified by PCR from *V. cholerae* O395 genomic DNA by using 5′ primers TcpHAA1 (5′-AAAAGAATTCCGC CTAGATAGTGTGTGA-CG-3′), RH11 (5′-GATCGGAATTCGCACGAGA CGAACACTGTC-3′), RH04 (5′-GATCGGAATTCAATTTCGATCTCCACT CCGG-3′), RH03 (5′-GACTGAATTCTTGAATTGAATAAGTTGGCC-3′), RT35 (5′-AAAACTCGAGGTGCGTGAATGTTACTCGTG-3′), and RH01 (5′-ATGCGAATTCCTTTCAATGCAAGTGTG-3′). The 3′ primer used for all these constructs was RTAA36 (5′-AAAAGGATCCACACGCACATTTAACC ACAC-3′). The *tcpA* promoter fragment for pRH98 was amplified from O395 chromosomal DNA by using primers RH12 (5′-GATCGAATTCAAAAAACA CAGCAAAAAATG-3′) and RH13 (5′-GACTGGATCCATGTAACTCCACC ATTTTAC-3′). The products were cloned into the *Eco*RI-*Bam*HI site of pRS415 and verified by PCR. *toxT* was provided on plasmid pTSS-5.

tcpA-lacZ fusions in pAJA3, pAJA2, pAJA1, pRH8, pRH98, pRH80, pCJ1.3, and pCJ10.4 were recombined onto λRS45 (59) and lysogenized into MC4100 to create strains RRH100, RRH115, RRH131, RRH113, RRH114, RRH101, RRH102, and RRH103, respectively. Single-copy lysogens were confirmed by PCR (53). pTSS-5 was introduced into each strain by calcium chloride transformation.

Plasmid pRH81 was constructed in the following manner. The *toxT* gene was amplified from O395 chromosomal DNA by using primers RH07 (5′ AGCCAT GGCACACCACCACCACCACCACATGATTGGGAAAAAATCTTTTC-3′), which adds a six-His tag on the N-terminal end, and RH08 (5′-AGCTTCTAG ACCCAAAATCAGTGATACAATCG-3′). The product was cloned into the *Nco*I-*Xba*I site of pBAD22 and confirmed by PCR and sequencing.

Plasmids pRH170 and pRH171 were constructed in the following manner. For pRH170, the entire *rpoA* gene was amplified from O395 chromosomal DNA by using primers RH17 (5′-GATCCTGCAGTTATCTTCAGCGATTGACG-3′) and RH19 (5′-GATCGGATCCCGAACAATTGATCGTCGAGC-3′). The product was cloned into the *Pst*I-*Bam*HI site of pMMB66EH and confirmed by both PCR and sequencing. For pRH171, primers RH17 and RH18 (5′-GATCC TGCAGTTATTACTCGTGCTGGTTCTTAAGATCTACGAACGCATCC-3′) were used to amplify the truncated *rpoA* gene (amino acids 1 to 235) from O395 chromosomal DNA. Primer RH18 encodes a five-amino-acid polar linker (KNQHE), followed by two ochre stops at the 5′ end. The linker was added for stabilization purposes (39, 52). The product was also cloned into the *Pst*I-*Bam*HI site of pMMB66EH and confirmed by PCR and sequencing. Both pRH170 and pRH171 were introduced into *V. cholerae* strains by mating (64) from strain SM10λ*pir*.

β-Galactosidase assays. All β-galactosidase assays (40) were carried out after growth in LB medium (pH 6.5) at 30°C. Strains carrying *tcpA-lacZ* fusions were assayed following overnight growth with aeration, while assays with *toxT-lacZ* fusion strains were performed by using mid-log-phase cultures. β-Galactosidase activity was expressed in Miller units (40).

Random PCR mutagenesis. Random PCR mutagenesis was carried out as previously described by Cadwell and Joyce (5) and Fromant et al. (16). Specifically, for the method of Cadwell and Joyce, the PCR mixtures contained 7 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dTTP, 1 mM dCTP, 0.5 mM $MnCl_2$,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
E. coli strains		
CC118	*araD139* Δ*(ara leu)7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	37
MC4100	*F*$^-$ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	58
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (λ*pir*)	43
AA60	CC118, pTSS-5	This study
AJA1	CC118, pAJA1	This study
AJA2	CC118, pAJA2	This study
AJA3	CC118, pAJA3	This study
RRH26	CC118, pRH8	This study
RRH72	CC118, pRH72	This study
RRH81	CC118, pRH81	This study
RRH82	CC118, pRH82	This study
RRH98	CC118, pRH98	This study
RRH100	MC4100 (λ*tcpA-lacZYA*) (−458/+2)	This study
RRH102	MC4100 (λ*tcpA-lacZYA*) (−162/+2), T to A at −38	This study
RRH103	MC4100 (λ*tcpA-lacZYA*) (−162/+2), A to T at −50	This study
RRH113	MC4100 (λ*tcpA-lacZYA*) (−95/+2)	This study
RRH114	MC4100 (λ*tcpA-lacZYA*) (−63/+72)	This study
RRH115	MC4100 (λ*tcpA-lacZYA*) (−162/+2)	This study
RRH131	MC4100 (λ*tcpA-lacZYA*) (−118/+2)	This study
V. cholerae strains		
O395 Sm	Classical Ogawa, Sm^r	63
CG842	O395 Sm Δ*lacZ*	8
MBN135	CG842 *tcpA-lacZ*	M. Nye
MBN142	MBN135 Δ*toxT*	M. Nye
MBN168	MBN135 Δ*toxT* Δ*hns*	M. Nye
RRH175	MBN135 (pRH170)	This study
RRH176	MBN135 (pRH171)	This study
RRH178	MBN142 (pRH170)	This study
RRH179	MBN142 (pRH171)	This study
RRH181	MBN168 (pRH170)	This study
RRH182	MBN168 (pRH171)	This study
MBN032	CG842 *toxT-lacZ*	49
RRH197	MBN032 (pRH170)	This study
RRH201	MBN032 (pRH171)	This study
Plasmids		
pTSS-5	pACYC184 *toxT*$^+$, Cm^r	3
pRS415	*lacZYA* transcriptional fusion vector, Ap^r	59
pAJA1	pRS415::(*tcpA-lacZYA*) (−118/+2)	This study
pAJA2	pRS415::(*tcpA-lacZYA*) (−162/+2)	This study
pAJA3	pRS415::(*tcpA-lacZYA*) (−458/+2)	This study
pRH8	pRS415::(*tcpA-lacZYA*) (−95/+2)	This study
pRH72	pRS415::(*tcpA-lacZYA*) (−230/+2)	This study
pRH82	pRS415::(*tcpA-lacZYA*) (−310/+2)	This study
pRH98	pRS415::(*tcpA-lacZYA*) (−63/+72)	This study
pCJ1.3	pRS415::(*tcpA-lacZYA*) (−162/+2), T to A at −38	This study
pCJ10.4	pRS415::(*tcpA-lacZYA*) (−162/+2), A to T at −50	This study
pBAD22	Expression plasmid, Ap^r	21
pRH81	pBAD22 six-His *toxT*	This study
pMMB66EH	Expression plasmid, Ap^r	17
pRH170	Full-length *rpoA* in pMMB66EH	This study
pRH171	*rpoA*Δ235 in pMMB66EH	This study

and 5 U of *Taq* polymerase. For the method of Fromant et al., the PCR mixtures contained 4.2 mM $MgCl_2$, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, 3.4 mM dATP, and 5 U of *Taq* polymerase. Each PCR consisted of 30 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min with primers RH03 and RTAA36 (see above). pAJA2 plasmid DNA was used as a template. Five separate pools were generated with each method. Pooled PCR products were purified on spin columns (Qiagen), digested with *Eco*RI and *Bam*HI, gel purified, and ligated into pRS415 to create *lacZ* fusions. The resulting pools of mutant constructs were electroporated into *E. coli* strain AA60, which carries *toxT* on plasmid pTSS-5. Colonies that appeared pink and shiny on 1% lactose–MacConkey agar plates were chosen for further study. Plasmid DNA was isolated from mutants by using Qiagen kits and was backcrossed into AA60 to confirm the phenotype. PCR was used to verify the presence of the desired insert. Inserts were sequenced by using automated fluorescence dye sequencing (Molecular Core Facility, Dartmouth Medical School).

Purification of ToxT, SDS-PAGE, and immunoblotting. The *toxT* gene was inserted into pBAD22 under control of the arabinose-inducible promoter p*araBAD*, and this construct was transformed into *E. coli* CC118. The resulting strain, RRH81, was grown to the mid-log phase at 30°C, and then arabinose was added to a final concentration of 0.02% and the strain was induced for 4 h at 30°C. Cell pellets were resuspended in extraction buffer (50 mM sodium phosphate, 300 mM NaCl; pH 7.0) and lysed by passage through a French pressure cell two or three times. Following a clarifying spin at 10,000 rpm in a Sorvall SS34 rotor for 20 min, supernatant and pellet fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Supernatants containing His-tagged ToxT were passed over Talon metal affinity resin (Clontech), and after extensive washing, ToxT was eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole). Column fractions were analyzed by SDS-PAGE, and fractions containing ToxT were pooled and dialyzed overnight against TEN buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA

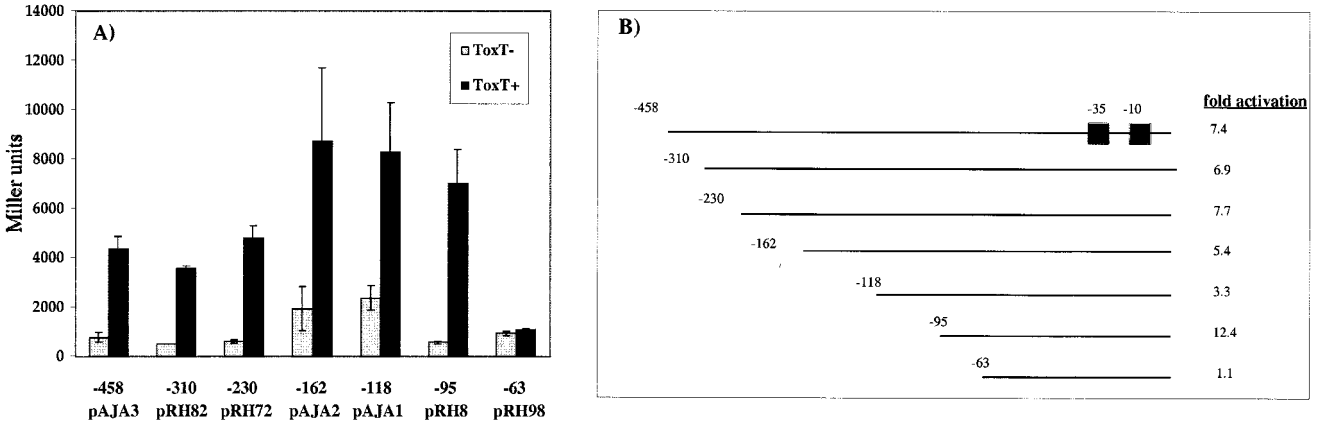


FIG. 1. ToxT-dependent activation of *tcpA-lacZ* promoter deletions in *E. coli*. (A) Strains with no ToxT (shaded bars) or strains with the ToxT-expressing plasmid pTSS-5 (solid bars) were grown overnight in LB medium (pH 6.5) at 30°C. Promoter deletions are carried on plasmid pRS415. The values are averages for at least two independent experiments. (B) *tcpA-lacZ* deletion series. Most promoter deletions are within a promoter fragment that extends to position +2 relative to the start of transcription; the only exception is the −63 deletion, which extends to +72. The level of activation in the presence of ToxT is indicated for each deletion.

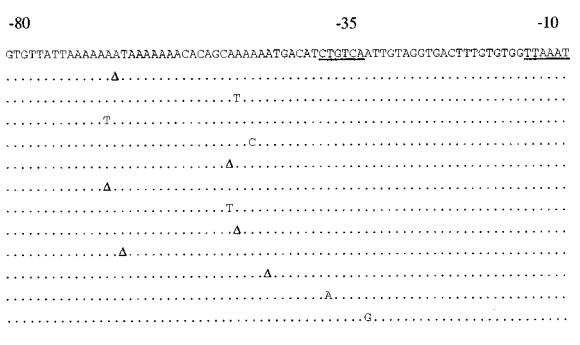
[pH 8]) containing 1 mM dithiothreitol. Protein concentrations were determined by the bicinchoninic acid procedure (Pierce). His-tagged ToxT in crude extracts and as pure protein was visualized by immunoblotting as follows. Proteins were transferred to nitrocellulose membranes, probed with anti-TetraHis antibody (Qiagen), and visualized by using ECL detection reagents (Amersham Pharmacia). Protein was stored at −70°C in 10% glycerol.

Gel mobility shift assays. PCR products of *tcpA* promoter regions were end labeled by using digoxigenin (DIG) (Gel Shift kit; Roche). The −95/+2, CJ10.4, CJ3.3, CJ2.6, and F4.2 probes were amplified from *V. cholerae* O395 chromosomal DNA or plasmids by using primers RH01 and RTAA36, the −63/+2 probe was amplified by using primers RH12 and RTAA36, and the −43/+2 probe was amplified by using primers RH16 (5′-GATCGAATTCGACATCTG TCAATTGTAGGT-3′) and RTAA36. The site I probe (probe −162/−51) was amplified from chromosomal DNA by using primers RH24 (5′-GTACTGCTG TGTTTTTTTATTTTTTTAATAAC-3′) and RH03. Probes were purified on agarose gels and extracted by using Gel Extraction kits (Qiagen). One microgram of probe was used for each labeling reaction. Labeled probe was resuspended in TEN buffer to a final concentration of 5 to 10 ng/μl. Five nanograms of labeled probe plus various amounts of pure His-tagged ToxT were used for each binding reaction. Also, 500 ng of calf thymus nonspecific competitor DNA was added to each reaction mixture. Binding reactions were carried out in binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 200 μg of bovine serum albumin per ml, 10% glycerol), and the reaction mixtures were incubated at 30°C for 30 min. Loading buffer without bromphenol blue was added, and samples were loaded onto a preelectrophoresed 6% native polyacrylamide gel. Gels were electrophoresed in 1× Tris-borate-EDTA at 120 V and 4°C. DNA was transferred to a positively charged nylon membrane (Roche) by electroblotting, cross-linked by using a UV Stratalinker (Stratagene), and visualized with an anti-DIG antibody by chemiluminescent detection by using reagents provided in the DIG kit (Roche).

RESULTS

Deletion series of the *tcpA* promoter region defines a minimal region required for ToxT-dependent activation. ToxT has previously been shown to directly activate *tcpA* expression with an *E. coli* host (3, 12). In order to delineate the *cis* requirements for activation by ToxT, a deletion series encompassing a region from −458 to −63 of the *tcpA* promoter was constructed in the cloning vector pRS415, which carries a promoterless *lacZ* gene downstream of the cloning site. The resulting *tcpA-lacZ* fusion constructs were transformed into *E. coli lacZ* deletion strain CC118, and *toxT* was provided on the pACYC184 derivative pTSS-5. The ability of ToxT to activate each *tcpA* promoter deletion was examined by determining the induction value for activated levels ($ToxT^+$) compared with basal levels ($ToxT^-$) for each deletion construct. As shown in Fig. 1, ToxT was able to activate transcription for the entire deletion series except pRH98, which is deleted to position −63 relative to the transcriptional start site of *tcpA*. This suggests that ToxT binds to a region of the *tcpA* promoter between positions −95 and +2, perhaps either upstream of or encompassing position −63. This region is extremely A rich. The promoter fusions upstream of −162 showed decreased levels of basal transcription compared to the levels of shorter fusions, indicating that there was a possible repressor binding site upstream of this position. The histone-like nuclear structuring protein H-NS has been shown to repress transcription of *tcpA* (49), and it has been proposed that this protein binds to one or more sites in this region of the promoter. ToxT partially overcomes the negative effect of H-NS at *tcpA* and *ctxA* (49).

Point mutations in the *tcpA* promoter that affect activation by ToxT cluster in three regions. To further define the location of the ToxT binding site at the *tcpA* promoter, PCR mutagenesis was carried out to create random point mutations in the promoter region. The region from −162 to +2 of the *tcpA* promoter was chosen as the template for mutagenic PCR since it included the region from −95 to −63, as well as flanking regions that might influence activation. Five separate pools of mutant PCR products were generated by the two procedures described in Materials and Methods. These products were cloned into pRS415 to create *lacZ* fusion constructs that were then transformed into AA60, an *E. coli* strain harboring *toxT* on pTSS-5. The transformants were plated on 1% lactose–MacConkey agar plates and grown at 30°C. Wild-type *tcpA-lacZ* fusion strains were dark red on these plates due to full activation by ToxT. White colonies lacked the promoter insert. Colonies harboring plasmids that were potentially defective for activation had a shiny pink appearance and white edges. Transformants that displayed this phenotype were restreaked on 1% lactose–MacConkey agar plates, and the presence of the *tcpA* promoter insert was confirmed by colony PCR. Plasmid DNA



plasmid	-ToxT	+ToxT	fold act.	
pAJA2	1945 (+/-900)	8715 (+/-3000)	5.4	← Wild type
pF4.3	275 (+/-68)	604 (+/-42)	2.2	ToxT activation in A/T rich regions (1st category)
pCJ10.4	339 (+/-9)	495 (+/-94)	1.5	
pCJ3.1	421 (+/-167)	675 (+/-140)	1.6	
pCJ4.3	480 (+/-147)	691 (+/-113)	1.4	
pCJ5.2	548 (+/-138)	851 (+/-105)	1.5	
pF1.1	564 (+/-83)	1213 (+/-34)	2.1	
pCJ2.8	980 (+/-294)	1874 (+/-25)	1.9	
pCJ6.3	1016 (+/-48)	1460 (+/-154)	1.4	
pF4.2	1364 (+/-449)	2416 (+/-418)	1.8	
pCJ8.5	1707 (+/-10)	2261 (+/-122)	1.3	
pCJ1.3	359 (+/-129)	1343 (+/-29)	3.7	Basal level in/or near −35 (2nd category)
pCJ2.3	769	8360	10.9	

FIG. 2. Sequences of single-point mutants with mutations in the region from −80 to −30, aligned with the sequence of the wild-type *tcpA* promoter. Base pair changes or deletions are shown; wild-type bases are indicated by dots. Strains carrying the *tcpA-lacZ* fusions on plasmid pRS415 with no ToxT or with the ToxT-expressing plasmid pTSS-5 were grown overnight in LB medium (pH 6.5) at 30°C. The β-galactosidase activity (in Miller units) and the level of activation (act.) in the presence of ToxT are shown for each strain. The values are averages for at least two independent experiments.

was isolated and backcrossed into AA60 to confirm that the activation defect was linked to the *tcpA* promoter-containing plasmid. β-Galactosidase assays were performed on the mutant strains, and the level of activation by ToxT for each mutant was compared to that of the wild-type strain. The mutants could be separated into two categories. The mutants in the first category showed an overall decreased level of transcription, particularly decreased activation by ToxT, which is the result expected for a mutation that disrupts activator function. The mutants in the second category showed a decreased level of basal transcription but retained wild-type levels of activation by ToxT, as expected for a mutation which disrupts RNA polymerase-DNA interactions. The majority of the mutants fell into the first category, but two mutants that are discussed below fell into the second category. It was found that for all of the mutants in the first category, ToxT was able to activate only one- to twofold compared with basal levels of transcription, compared to the fivefold activation observed for the wild type and for mutants in the second category.

Twenty representative mutants were sequenced, and their sequences were aligned with the wild-type sequence for the region. Seven of the mutants had a single base pair substitution or deletion, while the other 13 mutants had two or more base pair substitutions or deletions. The point mutations clustered in three distinct regions: the −35 region, a region centered at −60, and an upstream region centered at −117. No single mutations in the −117 region that did not have an accompanying mutation around −60 or −35 were isolated in our mutagenesis experiments, suggesting that the region centered at −117 is not in and of itself critical for activation by ToxT. This hypothesis is consistent with the results of the deletion analysis. Figure 2 shows the sequences of 12 of the mutants that have single mutations in either the −60 or −35 region. Ten of the mutants shown in Fig. 2 fall into the first category of mutants with decreased levels of activation by ToxT. All 10 of these mutants have single base pair substitutions or deletions in the region centered at −60, and ToxT is able to activate only one- to twofold compared with the basal levels, compared to the fivefold activation by the wild type. The mutations around −60 cluster in two regions separated by more than 10 bp, suggesting that they may define two distinct sites required for ToxT function. The single base pair deletions may also affect the spacing and rotation of these sites with respect to each other or the RNA polymerase binding site. Two of the mutants shown in Fig. 2 belong in the second category of mutants with decreased levels of basal transcription and wild-type levels of activation by ToxT. One of these mutants, CJ1.3, was found to have a single point mutation within the putative −35 region. The base pair change from T to A alters the sequence of the putative −35 hexamer further from the consensus TTGACA sequence. This mutant showed decreased levels of both basal and activated transcription compared to the wild-type levels, but the level of activation by ToxT was comparable to that of the wild type (3.7-fold versus 5-fold) (Fig. 2). Because the mutation falls in the putative −35 region and has only a minor effect on ToxT-mediated activation, we hypothesized that this point mutation affects RNA polymerase binding and does not greatly affect ToxT activity. The other mutant in the second category, CJ2.3, has only a defect in the basal level of transcription. ToxT is able to activate to wild-type levels (8,360 U) (Fig. 2). This mutant has a single base pair change, A to G at −33, within the putative −35 consensus region, which alters the sequence further from the consensus sequence. Since the only defect is a defect in basal transcription, we hypothesized that the point mutation of this mutant, like the CJ1.3 mutation, also affects the RNA polymerase binding site and the presence of ToxT is able to overcome this defect and provide full levels of activated transcription.

Analysis of a single T-to-C point mutation at −117 and creation of an internal deletion of the region from −140 to −95 allowed us to determine that the upstream region centered at −117 is not required for activation by ToxT (data not shown). The region closer to the transcriptional start site, centered at −60, is essential for activation by ToxT, as shown by the promoter deletion series and the random point mutations.

ToxT activation of single-copy chromosomal *tcpA* promoter fusions in *E. coli*. All of the experiments described above were carried out with multicopy plasmids in *E. coli*. In order to confirm that the results obtained were not due to alterations in plasmid supercoiling or multicopy effects, several representative *tcpA-lacZ* fusions were integrated into the *E. coli* chromosome as λ lysogens for further analysis. These included the

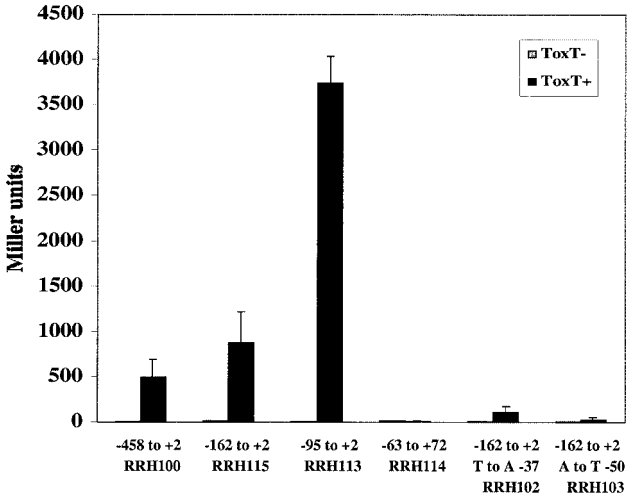


FIG. 3. ToxT-dependent activation of representative *tcpA-lacZ* promoter constructs as λ lysogens integrated into the *E. coli* chromosome. Strains with no ToxT or strains with the ToxT-expressing plasmid pTSS-5 were grown overnight in LB medium (pH 6.5) at 30°C. The values are averages for at least two independent experiments.

construct from −458 to +2 (pAJA3), the construct from −162 to +2 (pAJA2), the construct from −95 to +2 (pRH8), and the construct from −63 to +72 (pRH98). A single point mutation in the −35 region (CJ1.3) and a single point mutation at −50 in the putative ToxT binding site (CJ10.4) were also used. Single copies of these six constructs were integrated into the chromosome of *E. coli* MC4100 as λRS45 derivatives (59). ToxT was provided from plasmid pTSS-5, and β-galactosidase assays were performed. The overall trends followed what was observed with multicopy plasmids (Fig. 3). ToxT-dependent activation was lost with the fusion at −63 to +72. ToxT was not able to activate at levels significantly above basal levels when the A-to-T substitution at −50 was present but was able to activate in the strain containing the T-to-A substitution at −38.

Similar motifs at other ToxT-regulated promoters in *V. cholerae*. Data obtained with the deletion series and random point mutations suggest that the ToxT binding site at the *tcpA* promoter is located in an A-rich region from −82 to −45 relative to the transcriptional start site. Other ToxT-activated promoters were examined for similar motifs. An alignment of the putative −10 regions of the *tcpA*, *tcpI*, *tagA*, and *ctxA* promoters is shown in Fig. 4. The first three promoters, *tcpA*, *tcpI*, and *tagA*, are all very A rich. The *tcpI* promoter closely resembles *tcpA* in that there is a long A tract located around −65 relative to the transcriptional start site and a shorter A tract located closer to the −35 region. The *tagA* promoter has only one A tract, and it is located proximal to the −35 region. The similarity among these three ToxT-regulated promoters, together with the results of the genetic analysis of the *tcpA* promoter, suggests that ToxT may interact with these A-rich regions. The sequence upstream of *ctx* does not resemble the sequence upstream of *tcpA*, *tcpI*, or *tagA*; however, regulation at the *ctxA* promoter is much different from regulation at the other three promoters in that *ctxA* is regulated by both ToxT and the membrane-bound activator ToxR (12, 42, 49).

His-tagged ToxT binds directly to the *tcpA* promoter in vitro. The ability of ToxT to interact with the region of the *tcpA* promoter determined by genetic means was confirmed by gel shift analysis by using six-His-tagged ToxT. Purified His-tagged ToxT was used in gel mobility shift assays with DIG-labeled fragments of the *tcpA* promoter, as described in Materials and Methods. As shown in Fig. 5, ToxT was able to bind to and shift the region of the *tcpA* promoter from −95 to +2, which contains both of the A-rich regions identified by the deletion series and point mutations. ToxT was not able to shift the region of the *tcpA* promoter from −63 to +2, in which one-half of the promoter-distal A tract is removed. This is consistent with the data described above that show that this region is unresponsive to transcriptional activation by ToxT (Fig. 1). These results suggest that ToxT requires the promoter-distal A tract to bind and activate and cannot bind to the promoter-proximal A tract alone. ToxT also does not bind to a nonspecific probe encompassing the region from +1 to +130 of the *tcpP* promoter (Fig. 5), further demonstrating that the promoter-distal site-dependent binding is specific.

Next we wished to examine whether the lack of activation resulting from various point mutations in the *tcpA* promoter was due to a defect in ToxT binding. CJ3.3 has two mutations in the promoter-distal site and one mutation in the promoter-proximal site, CJ2.6 has one mutation in the promoter-distal site and one mutation in the promoter-proximal site, and F4.2 has one mutation in the promoter-distal site (Fig. 6A). ToxT was not able to detectably bind to a probe containing the mutations (Fig. 6B), suggesting that these point mutations are activation defective because they are binding defective. To specifically assess the role of the promoter-proximal site in binding, we used CJ10.4, which has an A-to-T mutation at −50 in this A tract. ToxT was able to bind to this probe, but with less avidity than to a wild-type probe (Fig. 6C), which is consistent with the decreased activation of this mutation (Fig. 2). To further investigate the relative roles of the two A tracts in ToxT binding, a probe was constructed from −162 to −51 containing the entire promoter-distal site but only one-half of

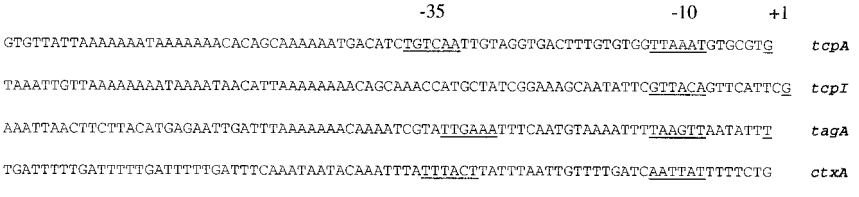


FIG. 4. Alignment of the *tcpA* promoter with other *V. cholerae* promoters regulated by ToxT. Sequences were aligned by using the −10 consensus sequence. The *tcpI* start site has been described previously (47), as have the *tcpA* start site (3) and the *ctxA* start site (51). The *tagA* start site is hypothetical.

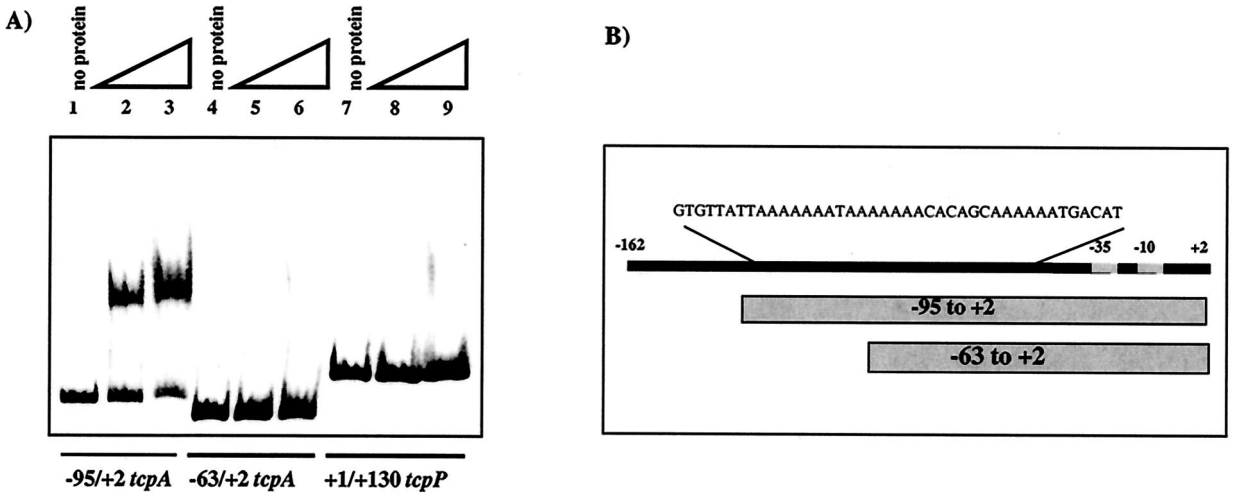


FIG. 5. Six-His-tagged ToxT binds to *tcpA* promoter fragments in gel mobility shift assays. (A) Lanes 1 to 3, −95/+2 *tcpA* probe (lane 1, no protein; lane 2, 150 ng [4.6 pmol] of six-His-tagged ToxT; lane 3, 300 ng [9.3 pmol] of six-His-tagged ToxT); lanes 4 to 6, −63/+2 *tcpA* probe (lane 4, no protein; lane 5, 150 ng [4.6 pmol] of six-His-tagged ToxT; lane 6, 300 ng [9.3 pmol] of six-His-tagged ToxT); lanes 7 to 9, +1/+130 *tcpP* probe (lane 7, no protein; lane 8, 150 ng [4.6 pmol] of six-His-tagged ToxT; lane 9, 300 ng [9.3 pmol] of six-His-tagged ToxT). (B) Diagram showing the positions of *tcpA* promoter fragments used in the gel shift analysis. The −95/+2 probe includes both A tracts, while the −63/+2 probe includes one-half of the distal A tract and the entire proximal A tract.

the promoter-proximal site. The inability of ToxT to produce a detectable shift of this probe suggests that ToxT is not able to bind to the distal site in the absence of the proximal site (Fig. 6C). This, coupled with the results of the analysis of the distal site presented above, suggests that ToxT requires the presence of both A tracts for binding.

ToxT requires αCTD of RNA polymerase to activate transcription at *tcpA*. It has been demonstrated that many AraC family members require the αCTD of RNA polymerase to activate transcription at their respective promoters (27, 30, 32, 38, 56). We tested the requirement for αCTD for ToxT-dependent transcriptional activation at *tcpA* by performing a genetic analysis similar to that described by Holcroft and Egan (27). The region of the *V. cholerae rpoA* gene corresponding to residues 1 to 235 was amplified from O395 chromosomal DNA and cloned under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. This resulted in a truncated α subunit that was missing the C-terminal domain. We hypothesized that when the truncated α subunit is overexpressed in *V. cholerae*, it assembles with RNA polymerase subunits expressed from the chromosome and acts as a dominant negative allele with respect to promoters at which αCTD has a role in expression. This hypothesis is based on previous experiments performed with *E. coli* (23). A full-length *rpoA* construct was also constructed as a control. Each of the *rpoA* constructs was introduced into *V. cholerae* strains carrying a chromosomal *tcpA-lacZ* fusion. In a wild-type background, overexpression of the truncated α subunit resulted in an 11-fold decrease in *tcpA-lacZ* expression compared to the expression when a full-length α subunit was overexpressed (Fig. 7A). This effect was not seen in a Δ*toxT* background or a Δ*toxT* Δ*hns* background, which was used to elevate the level of *tcpA* expression in the Δ*toxT* background (Fig. 7A). These results suggest that only ToxT-dependent transcription and not basal transcription of the *tcpA* promoter depends upon the α subunit of RNA polymerase.

To determine whether the 11-fold defect in transcription of *tcpA-lacZ* was due to a direct effect of the truncated αCTD at the *tcpA* promoter and not to the effects of other promoters upstream in the regulatory cascade, we expressed ToxT from the αCTD-independent *tetR* promoter using plasmid pTSS-5. These experiments were carried out with *E. coli* since the *tetR* promoter is not expressed well in *V. cholerae*. *E. coli* λ*tcpA-lacZ* strain RRH115 (−162/+2) carrying either full-length *rpoA* or Δ235 *rpoA* on pMMB66EH and pTSS-5 as a source of ToxT was assayed by performing β-galactosidase assays. We found that when ToxT was expressed from an αCTD-independent promoter, there was an approximately fourfold defect in *tcpA-lacZ* transcription due to overexpression of Δ235 *rpoA* (Fig. 7B). These results also suggest that ToxT interacts with αCTD to activate transcription at *tcpA*. Due to the difference between the 11-fold defect in *V. cholerae* when ToxT was expressed from its own promoter and the 4-fold defect in *E. coli* when ToxT was expressed from the *tetR* promoter, we introduced the *rpoA* constructs into a *V. cholerae* strain carrying a chromosomal *toxT-lacZ* fusion. We observed a twofold defect when the dominant negative allele was overexpressed (data not shown). These results suggest that αCTD has a role at the *toxT* promoter, perhaps through an interaction with ToxR or TcpP.

DISCUSSION

AraC family members can be divided into different categories based on the types of genes that they regulate, such as the genes involved in sugar catabolism, stress response, and bacterial pathogenesis. It has been shown that family members involved in the regulation of sugar catabolism, such as AraC itself, function as dimers and have both a C-terminal DNA

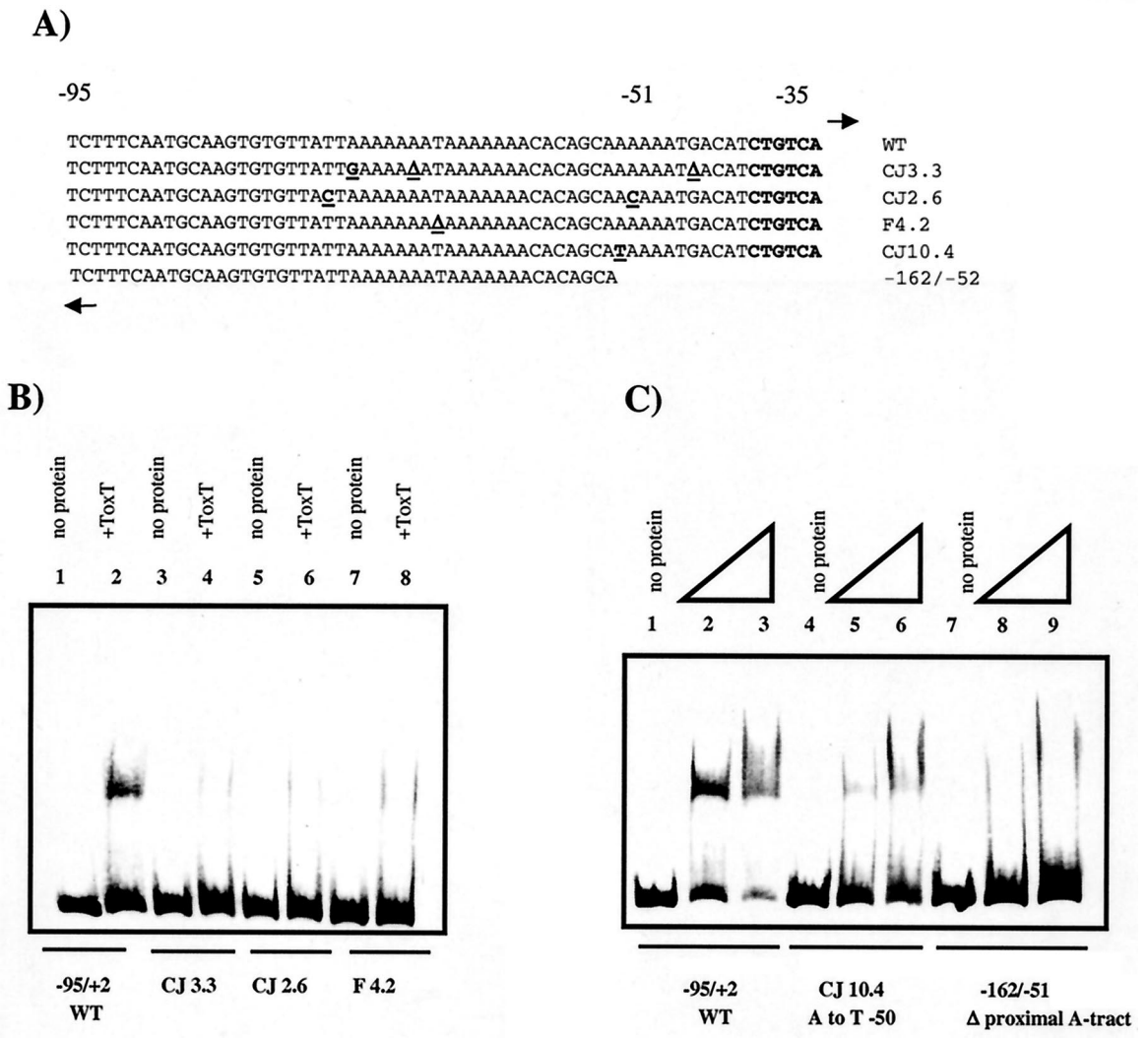


FIG. 6. Gel mobility shift assays with various *tcpA* promoter mutants. (A) Sequences of mutant probes used in binding studies. Base pair changes are underlined. Most of the probes are from −95 to +2; the only exception is the probe from −162 to −51. WT, wild type. (B) Lanes 1 and 2, −95/+2 *tcpA* probe (lane 1, no protein; lane 2, 150 ng [4.6 pmol] of six-His-tagged ToxT); lanes 3 and 4, CJ3.3 probe (lane 3, no protein; lane 4, 150 ng [4.6 pmol] of six-His-tagged ToxT); lanes 5 and 6, CJ2.6 probe (lane 5, no protein; lane 6, 150 ng [4.6 pmol] of six-His-tagged ToxT); lanes 7 and 8, F4.2 probe (lane 7, no protein; lane 8, 150 ng [4.6 pmol] of six-His-tagged ToxT). (C) Lanes 1 to 3, −95/+2 *tcpA* probe (lane 1, no protein; lane 2, 150 ng [4.6 pmol] of six-His-tagged ToxT; lane 3, 300 ng [9.3 pmol] of six-His-tagged ToxT); lanes 4 to 6, CJ10.4 probe (lane 4, no protein; lane 5, 150 ng [4.6 pmol] of six-His-tagged ToxT; lane 6, 300 ng [9.3 pmol] of six-His-tagged ToxT); lanes 7 to 9, site I probe (lane 7, no protein; lane 8, 150 ng [4.6 pmol] of six-His-tagged ToxT; lane 9, 300 ng [9.3 pmol] of six-His-tagged ToxT).

binding domain and an N-terminal dimerization domain. The N-terminal domain also functions to bind regulatory cofactors, such as sugar molecules. These proteins have binding sites that overlap the −35 position (class II promoters) (38). Family members such as Rob, MarA, and SoxS regulate genes involved in stress response and function as monomers. These proteins can have binding sites that are upstream of the −35 position (class I promoters) or overlap the −35 position (class II promoters) (30, 38). ToxT belongs to the subgroup of AraC homologs that regulate virulence gene expression. Less is known about this subgroup than about any other subgroup, but the members have been shown to activate transcription at both class I and class II promoters. The *tcpA* promoter appears to be a class I promoter since ToxT binds upstream of the −35 position. We propose that the primary binding site does not overlap the −35 position for the following reasons. A point mutation at −38 (CJ1.3) affects overall transcription but does not affect the level of activation by ToxT (Fig. 2), suggesting that this mutation interferes only with RNA polymerase binding. It has been shown that SoxS requires only the αCTD of RNA polymerase to activate transcription at promoters where the binding site does not overlap the −35 position (i.e., class I promoters) (30). We have shown that ToxT requires the RNA polymerase αCTD to activate transcription at *tcpA* (Fig. 7). Furthermore, a recent paper by Yu et al. showed that ToxT protects the region from −84 to −41 of *tcpA* in DNase I protection assays (69). This footprint does not overlap the putative −35 site and is consistent with the data presented here. Munson and Scott have recently demonstrated that the AraC homolog Rns from enterotoxigenic *E. coli* requires a

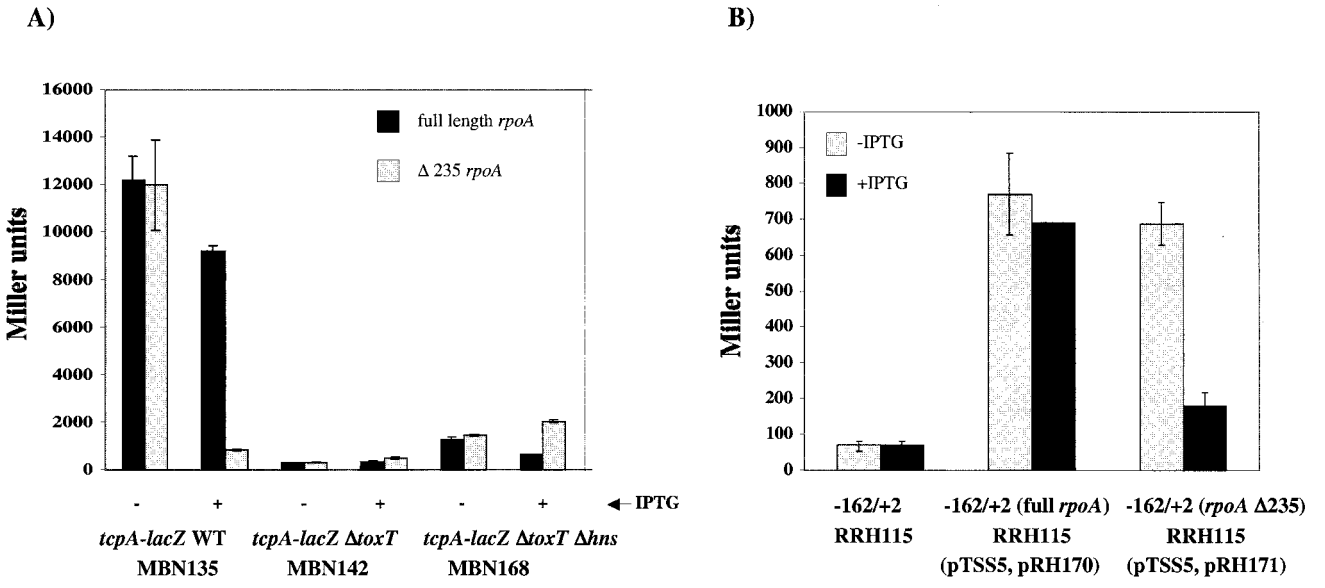


FIG. 7. Effects of dominant negative RNA polymerase alpha subunit on *tcpA-lacZ* expression in *V. cholerae* (A) and *E. coli* (B). (A) Cultures of MBN135 (*tcpA-lacZ*), MBN142 (*tcpA-lacZ* Δ*toxT*), or MBN168 (*tcpA-lacZ* Δ*toxT* Δ*hns*) carrying either pRH170 (full-length *rpoA*) or pRH171 (*rpoA* Δ235) were grown overnight in LB medium (pH 6.5) at 30°C with or without 0.04 mM IPTG. The values are averages for at least two independent experiments. WT, wild type. (B) Cultures of RRH115 (λ*tcpA-lacZ* −162/+2) carrying no plasmid or pTSS-5 and either pRH170 or pRH171 were grown overnight in LB medium (pH 6.5) at 30°C with or without 0.04 mM IPTG. The values are averages for at least two independent experiments.

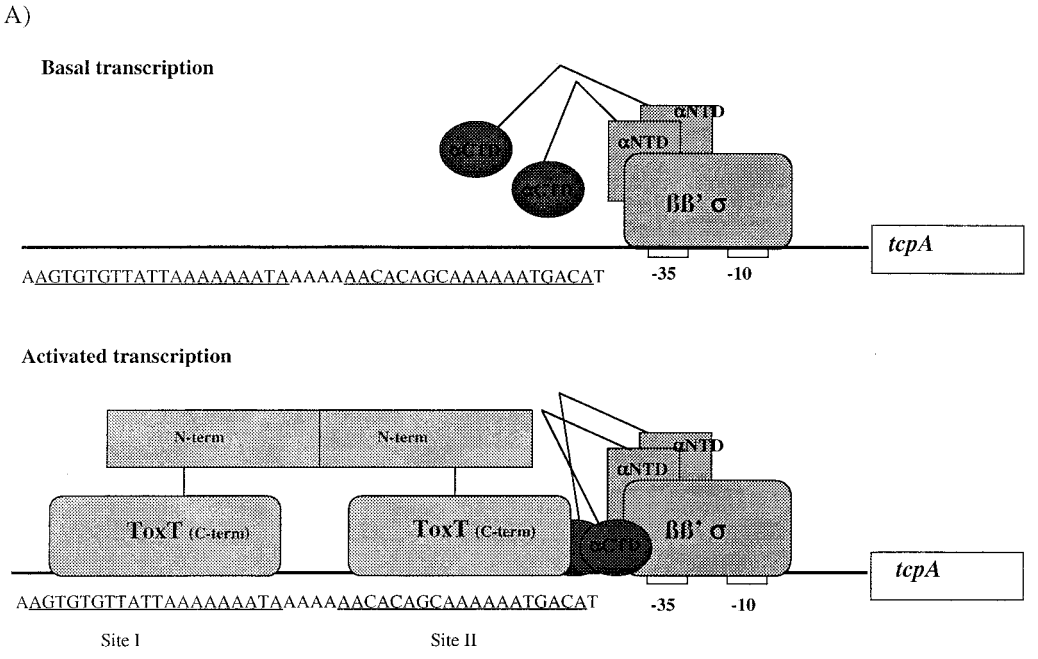
binding site downstream of the transcriptional start site to activate transcription at its own promoter (46). We have no indication that ToxT binds downstream of *tcpA*; in fact, our *tcpA-lacZ* fusions ended at +2, which suggests that ToxT does not require downstream sequences to activate expression from the *tcpA* promoter.

The binding site of ToxT at *tcpA* consists of two A-rich regions on the sense strand. Deletions of a single A residue or base pair substitutions in these A-rich regions and the surrounding DNA interfere with ToxT-dependent transcriptional activation and binding. There is no obvious dyad symmetry in the binding site, but AraC homologs tend to recognize asymmetric binding sites (18, 45). Two AraC homologs in particular have been shown to bind to A-rich DNA sequences. ExsA from *P. aeruginosa* binds to a consensus sequence (TXAAAAXA) located around −50 relative to the transcriptional start site (29). BfpT (PerA) from enteropathogenic *E. coli* requires an A-rich region from −85 to −46 of the *bfpA* promoter to activate transcription, and deletions of As in this region were found to negatively affect BfpT-dependent transcriptional activation (4). BfpT is particularly interesting because like ToxT, this protein regulates genes encoding a type IV pilus (bundle-forming pilus) (4).

Many transcriptional activators have been shown to interact with different subunits of RNA polymerase to activate transcription (67). There are data which suggest that various AraC homologs require the αCTD of RNA polymerase (1, 27, 30, 32, 38, 56) at class I promoters. In this paper we present data obtained by using a truncated alpha subunit which suggest that ToxT requires αCTD to activate transcription at *tcpA*. Our experiments also indicated that the αCTD does not seem to be required for basal transcription at *tcpA*. This implies that the A-rich sequence upstream of *tcpA* does not act as an UP element. UP elements are A-T-rich sequences that can bind αCTD and stimulate transcription independent of activator proteins (14, 19). UP elements consist of two subsites, a promoter-proximal subsite and a promoter-distal subsite. The proximal subsite is typically centered at −40 and has a consensus 5′-AAAAAARNR-3′ sequence, while the distal subsite is centered at −50 and has a consensus 5′-AWWWWWTTTT T-3′ sequence (14). The sequence of the promoter-proximal A tract loosely resembles that of a consensus UP element proximal site, but the tract is centered at −47, not at −40, and is therefore rotated around the DNA compared to a typical UP element. There is no distal site at *tcpA*. These observations, along with our dominant negative data, strongly suggest that there is no functional UP element at the *tcpA* promoter.

There is genetic evidence that there is an interaction between the AraC homolog RhaS and the sigma 70 subunit of RNA polymerase (1). Bhende and Egan (1) found a specific residue within RhaS, D241, that may be important for contacting sigma. This residue is conserved in many AraC family members (1, 18) but not in ToxT. Because ToxT does not have an Asp at position 241 and because its binding site does not overlap the −35 position (69), we propose that the mechanism of ToxT-dependent activation at *tcpA* does not involve an interaction with sigma. This remains to be determined experimentally. Some activators are known to contact the N-terminal domain of alpha, but an extensive genetic screening analysis performed by Egan et al. (13) suggested that AraC family members do not use this mechanism to activate transcription (13).

Our data begin to elucidate the mechanism of ToxT-dependent transcriptional activation at the *tcpA* promoter, a model of which is depicted in Fig. 8A. In our model, basal transcrip-



B)

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          Site I                    Site II
AAGTGTGTTATTAAAAAAATAAAAAAACACAGCAAAAAATGACATC      tcpA
TATTTAAATTGTTAAAAAAAATAAAATAACAATTAAAAAAAACAGC      tcpI
AAATTAACTTCTTACATGAGAATTGATTTAAAAAAACAAAATCGTA      tagA

-Ttaa-Tt-TtAaAaaA-A    At-acAa-aAAaAAaaaCa        consensus
```

FIG. 8. Model of transcriptional activation at *tcpA*. (A) During basal transcription, αCTD does not contact DNA. During activated transcription, a dimer of ToxT binds to site I (−82 to −64) and site II (−59 to −41). The ToxT molecule at site II recruits RNA polymerase through a mechanism involving the αCTD. See text for details. αNTD, alpha N-terminal domain. (B) Alignment of site I and site II at *tcpA*, *tcpI*, and *tagA*. Site I and site II at each promoter are underlined. The consensus sequence is shown at the bottom. An uppercase letter indicates that a base pair is present in all three promoters, and a lowercase letter indicates that base pair is conserved in two of the three promoters.

tion at *tcpA* is independent of αCTD since there does not appear to be a functional UP element. In order to activate transcription, ToxT binds to an A-rich region from −84 to −41, the region that ToxT protected in a DNase I footprint published recently by Yu and DiRita (69). While the footprint defines the general region where ToxT binds, in our study we used a mutational analysis to further investigate individual nucleotides important for ToxT binding and activation. The region bound by ToxT is approximately 40 bp long, which could encompass four major grooves. Based on the AraC model (24, 48), this provides sufficient space for one dimer of ToxT to bind. It is not known whether ToxT is able to dimerize, but we have found that mutations that delete either one or both of the C-terminal HTH domains have a dominant negative phenotype with respect to *tcpA* expression, indicating that there is functional multimerization of ToxT (unpublished data). These preliminary results suggest that ToxT can dimerize prior to binding DNA. The activation-defective point mutations define two small regions from −65 to −67 and from −46 to −51, and we propose that these regions represent two of the major grooves contacted by ToxT. These major grooves are not adjacent, further supporting the hypothesis that ToxT binds as a dimer. Our mutational analysis did not identify the other two major grooves contacted by ToxT, but the locations of the two major grooves that we did identify are consistent with two ~20-bp half-sites from −82 to −64 (site I) and from −59 to −41 (site II). This is similar to the AraC model (48). The ToxT molecule centered at site II, closer to the −35 region, should be in position to interact with RNA polymerase. We have demonstrated that ToxT requires the αCTD of RNA polymerase to activate transcription at *tcpA* (Fig. 7). It remains to be determined experimentally whether αCTD contacts DNA and/or ToxT. Figure 8B shows an alignment of the *tcpA* promoter with *tcpI* and *tagA*, two other *V. cholerae* promoters

that are regulated by ToxT. Similarly positioned A-rich motifs suggest that ToxT may bind to these promoters in a similar manner.

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