

## Flexibility of *Vibrio cholerae* ToxT in Transcription Activation of Genes Having Altered Promoter Spacing<sup>∇</sup>

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**Cholera, a severe diarrheal disease, is caused by ingestion of the gram-negative bacterium *Vibrio cholerae*. Expression of *V. cholerae* virulence factors is highly regulated at the transcriptional and posttranscriptional levels by a complex network of proteins and small noncoding RNAs. The direct activator of transcription of most *V. cholerae* virulence genes is the ToxT protein. ToxT binds to a 13-bp sequence, the toxbox, located upstream of genes in its regulon. However, the organization of toxboxes relative to each other and to the core promoter elements at different genes varies dramatically. At different ToxT-activated genes a single toxbox may be necessary and sufficient for full activation, or pairs of toxboxes organized as either inverted or direct repeats may be required for full activation. Although all toxboxes are located at positions consistent with a class I promoter architecture, the locations of toxboxes relative to the transcription start site also vary from gene to gene. To further assess the ability of ToxT to activate transcription from different configurations relative to the core promoter elements, we constructed promoter-*lacZ* fusions having altered spacing both between toxbox pairs and between the promoter-proximal toxbox and the  $-35$  box at five different ToxT-activated promoters. Our results suggest that that ToxT has remarkable flexibility in its positioning as a transcription activator and that different interactions between ToxT and RNA polymerase occur during transcription activation of promoters having different toxbox configurations.**

The gram-negative bacterium *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, continues to be a serious public health problem despite over 100 years of research on its pathogenesis. The current cholera pandemic, which began in 1961, continues unabated, and an estimated 5 million cases of cholera occur worldwide each year. Although over 200 known serogroups of *V. cholerae* have been identified, only the O1 and O139 serogroups are capable of causing pandemic cholera (34, 35).

Pandemic *V. cholerae* strains require two major virulence factors for colonization and pathogenesis. The first factor, cholera toxin, is an A<sub>1</sub>B<sub>5</sub>-type ADP-ribosylating toxin that is responsible for producing the voluminous watery diarrhea characteristic of cholera (8, 26). The genes encoding cholera toxin, *ctxAB*, are carried in the genome of a lysogenic bacteriophage, CTXΦ (40). The second major virulence factor is the toxin-coregulated pilus (TCP) (38), a type IV pilus that initiates microcolony formation and is required for intestinal colonization by *V. cholerae* (39). Genes encoding the TCP, which are in a long operon beginning with the *tcpA* pilin gene, are located in the *Vibrio* pathogenicity island (VPI) (22). In addition to its role as a colonization factor, the TCP is also the receptor for CTXΦ and thus allows horizontal transfer of *ctxAB* to nontoxicogenic *V. cholerae* carrying the VPI (40). In addition to the TCP-encoding genes, several other genes thought to be involved in pathogenesis are located in the VPI (1). These genes encode the accessory colonization factors (*acfA*, *acfB*, *acfC*, and *acfD*) (6, 17, 18), a putative lipoprotein

(*tagA*) (9), an aldehyde dehydrogenase (*aldA*) (32), and a putative methyl-accepting chemotaxis protein (*tcpI*) (10).

The expression of *V. cholerae* virulence genes is highly regulated. A cascade of both positive and negative transcription regulators is involved in initiating expression of all of the genes mentioned above (29). Furthermore, some gene products in the regulatory cascade are further controlled posttranscriptionally (30). Historically, the virulence regulon is referred to as the ToxR regulon. However, the direct transcriptional activator of *ctxAB*, the *tcpA* operon, the *acfABCD* genes, *aldA*, *tagA*, and *tcpI*, is a 32-kDa protein, ToxT. The combined actions of the transcription activators ToxR and TcpP are required for expression of ToxT (5,11–13, 23, 24).

ToxT is a member of the large AraC/XylS family of transcription regulators (14). The C-terminal 100 amino acids of ToxT form the AraC/XylS homology domain (7, 27) and likely bind to DNA, whereas the remaining 176 amino acids form an N-terminal domain that has no homology to any other protein based on BLAST and PSI-BLAST searches and protein threading programs. ToxT binds to a degenerate 13-bp DNA site, the toxbox, which is located upstream of all the genes in its regulon (41). The toxbox consists primarily of a T tract toward the 5' end and A/T-rich sequence in the 3' half. Mutagenesis of every base pair in the ToxT binding region upstream of *tcpA* indicated that as few as 5 bp and at most 7 bp in one 13-bp toxbox have sequence-specific roles in activation by ToxT (41). Furthermore, in addition to having sequence diversity, toxboxes also have diverse configurations relative to the core promoter elements (Fig. 1). Upstream of *aldA*, a single toxbox is required for transcription activation (42). Upstream of *tcpA*, two toxboxes configured as a direct repeat are required for transcription activation (41). Upstream of *tagA*, two toxboxes configured as an inverted repeat are required (42). Between

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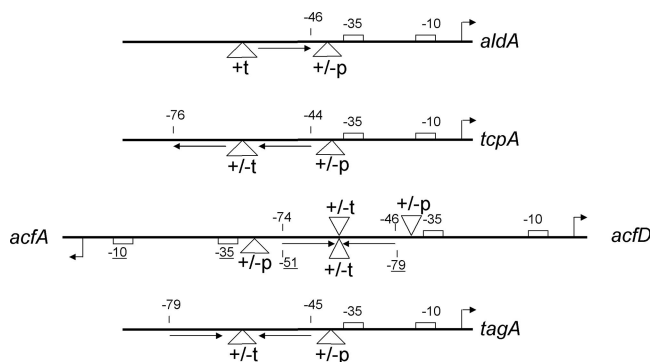


FIG. 1. Promoter architecture and design of spacing mutations. The numbers indicate distances from the transcription start site (indicated by a bent arrow). The numbers for the *acfA* promoter are underlined to distinguish them from the numbers for the *acfD* promoter. The boxes indicate the positions of  $-35$  and  $-10$  promoter elements. The arrows below the thick black lines indicate the positions of toxboxes at each promoter. The open triangles indicate the locations of insertion or deletion mutations. “+/-p” indicates that insertions or deletions were made between the promoter-proximal toxbox and the  $-35$  box, and “+/-t” indicates that insertions or deletions were made upstream of the toxbox (for *aldA*) or between the toxboxes.

*acfA* and *acfD*, two toxboxes configured as an inverted repeat are required for transcription activation of both genes (43). The *acfA-acfD* inverted repeat differs from the *tagA* inverted repeat both in the spacing between the toxboxes and in the spacing between the promoter-proximal toxbox and the  $-35$  box (Fig. 1).

Based on this diversity in DNA binding site configurations, we have proposed that ToxT binds to independent toxboxes as a monomer (41, 43). This hypothesis was tested by inserting 5 and 10 bp between either the *acfA* and *acfD* toxboxes or the *tcpA* toxboxes and assessing the occupancy of each toxbox using copper-phenanthroline (CP) footprinting. We found that ToxT bound to toxboxes regardless of their location relative to each other at promoters having either a direct repeat (*tcpA*) or an inverted repeat (*acfA-acfD*) configuration (41, 43). This strongly suggested that ToxT binds to individual toxboxes as an independent monomer. However, the insertions between toxboxes abrogated transcription activation by ToxT, suggesting that the spacing of toxboxes relative to each other is important for ToxT function. Furthermore, insertion of 5 or 10 bp between the promoter-proximal toxbox and the  $-35$  box at the *tcpA* promoter also abrogated transcription activation by ToxT (unpublished data), suggesting that the spacing of the toxboxes relative to the promoter is important for activation. There is evidence from domain swapping experiments that ToxT N-terminal domains can associate with each other to form dimers or multimers (33, 37), but full-length ToxT was not observed to dimerize in such an assay (37). It is possible that ToxT monomers associate with each other at some promoters and that this association is important for transcription activation.

In this work, we further assessed the importance of toxbox spacing for ToxT-mediated transcription activation. We constructed promoter-*lacZ* fusions having altered spacing between toxboxes at four different dual toxbox promoters and between the promoter-proximal toxbox and the  $-35$  box at five promoters. Our findings indicate that each promoter has an optimal

spacing for activation by ToxT that is equal to or close to the wild-type spacing, although the optimal spacing is different for different promoters. We propose that at least three different types of interactions between ToxT and RNA polymerase (RNAP) occur in transcription activation depending on the ToxT binding site position and orientation relative to the promoter and that these interaction types differ in their sensitivity to spacing alterations.

## MATERIALS AND METHODS

***V. cholerae* strains and plasmids.** The strains used experimentally in this work were classical *V. cholerae* strain O395 and derivatives of this strain. The strains were grown in LB medium at 37°C (overnight cultures) or in LB medium with a starting pH of 6.5 at 30°C (inducing conditions). The strains used for  $\beta$ -galactosidase assays were O395 and an O395  $\Delta$ *toxT* derivative (VJ740) carrying the previously specified promoter fusion in plasmid pTL61T (3). Spacing mutations were created using the splicing-by-overlap-extension technique (15, 16), and all promoter constructs were cloned between the HindIII and XbaI sites of pTL61T (25). Plasmid sequences were confirmed by the University of Michigan DNA sequencing core. Antibiotics were used at the following concentrations: ampicillin, 100  $\mu$ g/ml; and streptomycin, 100  $\mu$ g/ml. *V. cholerae* was transformed with plasmid DNA by electroporation using a Bio-Rad MicroPulser.

**DNA manipulation.** Plasmids were purified using a Promega Wizard Plus Miniprep kit. PCR was performed using *Taq* DNA polymerase (Denville Scientific) as specified by the manufacturer and an Eppendorf Mastercycler gradient. Restriction enzymes were purchased from New England Biolabs and were used as specified by the manufacturer.

**$\beta$ -Galactosidase assays.** Strains were cultured overnight in LB medium at 37°C and then subcultured using a 1/50 dilution in inducing medium and grown for 3 h at 30°C with vigorous aeration. The bacteria were then placed on ice, and 0.5 mg ml<sup>-1</sup> chloramphenicol was added.  $\beta$ -Galactosidase assays were performed using the basic procedure of Miller as previously described (31).

**CP footprinting.** CP footprinting was performed as previously described (41–43). Chemical cleavage was done in a gel after separation of free DNA and a bound ToxT-DNA complex by using an electrophoretic mobility shift assay. DNA was produced by PCR after one primer was end labeled with <sup>32</sup>P using T4 polynucleotide kinase (New England Biolabs). Polyhistidine-tagged ToxT was purified as previously described (45). The ratio of ToxT to DNA used was adjusted such that approximately 50% of the labeled DNA formed a bound complex with ToxT.

## RESULTS

**Design of spacing variants.** Previous work indicated that insertion of 5 bp and insertion of 10 bp between the toxboxes, resulting in rotation of approximately one half turn and one full turn of the DNA helix, respectively, at the *tcpA* and *acfA-acfD* promoters abrogated transcription activation by ToxT, although the toxboxes were still occupied by ToxT in DNA footprinting experiments (41, 43). Insertion of 5 bp and insertion of 10 bp between the promoter-proximal toxbox and the  $-35$  box at the *tcpA* promoter, resulting in rotation of approximately one half turn and one full turn of the DNA helix, respectively, between both toxboxes and the core promoter elements also abrogated activation by ToxT (unpublished data). Because there is natural variation in the positions of toxboxes at different promoters (Fig. 1), we constructed variant promoter regions in which the spacing either between toxboxes (+t or -t) or between the promoter-proximal toxbox and the  $-35$  box (+p or -p) was altered in 1-bp increments by either insertion or deletion (Fig. 1). The resulting promoters were fused to *lacZ* in pTL61T (25) and then tested for activation by ToxT by comparing  $\beta$ -galactosidase levels in classical *V. cholerae* strains having wild-type *toxT* or a *toxT* deletion.

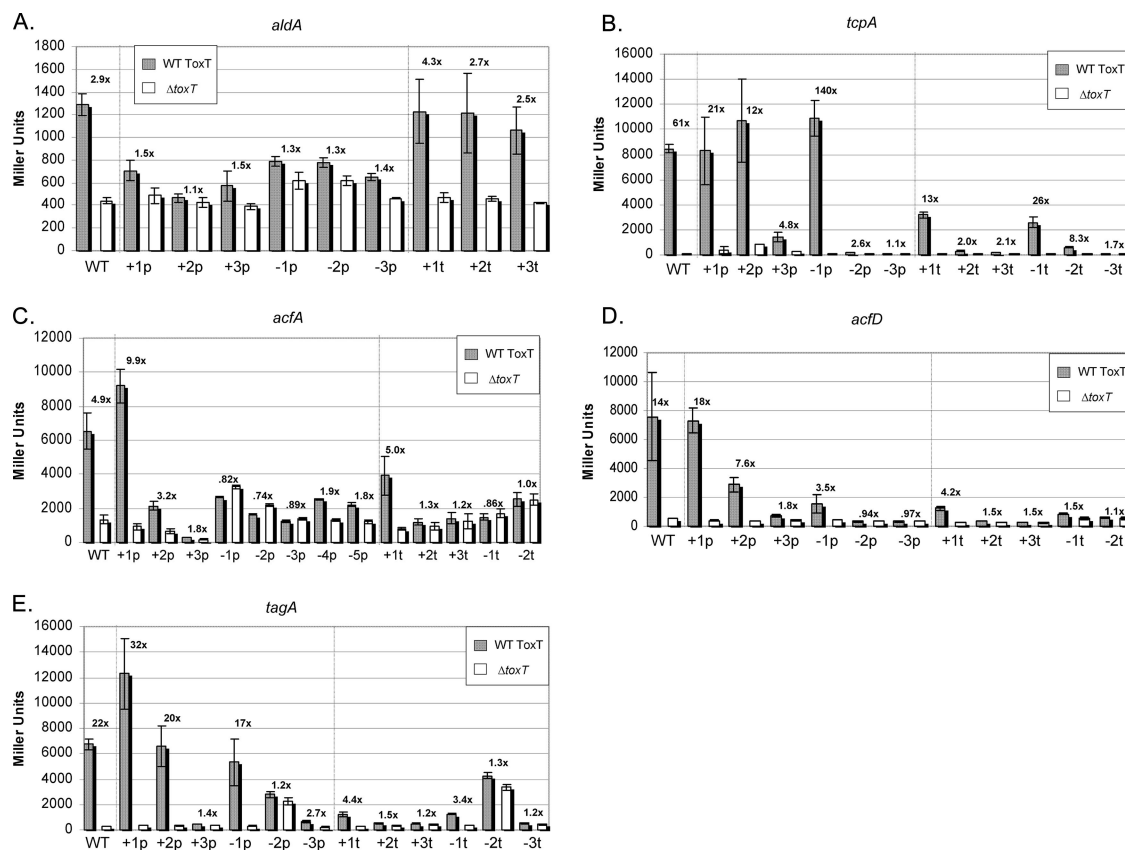


FIG. 2. Effects of promoter spacing mutations on  $\beta$ -galactosidase expression of promoter spacing mutants. The gray bars indicate the results for wild-type *toxT* *V. cholerae* strains (WT), and the open bars indicate the results for  $\Delta$ *toxT* strains. The number above each pair of bars indicates the difference in  $\beta$ -galactosidase expression between the wild-type *toxT* and  $\Delta$ *toxT* strains. The bars indicate the means of at least three separate experiments, and the error bars indicate standard deviations. (A) *aldA-lacZ* fusions. (B) *tcpA-lacZ* fusions. (C) *acfA-lacZ* fusions. (D) *acfD-lacZ* fusions. (E) *tagA-lacZ* fusions.

**Effects of spacing mutations on *aldA* activation.** The *aldA* promoter is the only known promoter in which a single toxbox is necessary and sufficient for activation by ToxT (42), although ToxT may act as a repressor at other single toxboxes (J. H. Withey, unpublished data). Therefore, insertions upstream of the toxbox should have no effect on the level of activation conferred by ToxT. As shown in Fig. 2A, this was indeed the case. However, alteration of the spacing between the toxbox and the  $-35$  box by even 1 bp abrogated activation of *aldA* transcription by ToxT. The *aldA* toxbox is in the orientation opposite that of toxboxes at similar locations in other promoters (at *aldA* the toxbox “points toward” the promoter [Fig. 1]), so these results suggest that a single toxbox in this orientation is extremely sensitive to spacing changes. *aldA* transcription is also relatively weakly activated by ToxT even with wild-type spacing of the toxbox and promoter (Fig. 2A).

**Effects of spacing mutations on *tcpA* activation.** Two toxboxes in a direct repeat configuration are required for activation of the *tcpA* promoter by ToxT (41). Transcription of *tcpA* is very strongly activated by ToxT, as might be expected given the crucial role of the TCP in pathogenesis (39). In contrast to what we observed for the *aldA* promoter, activation of *tcpA* transcription by ToxT was observed despite considerable changes in spacing between the proximal toxbox and the  $-35$

box. Constructs having up to 3-bp insertions and a 1-bp deletion were significantly activated by ToxT (Fig. 2B). Insertion of 4 or 5 bp abrogated activation of *tcpA* by ToxT (data not shown). The difference in spacing between the *tcpA* +3p and *tcpA*  $-1p$  constructs would be rotation of approximately  $137^\circ$  and a distance of 1.32 nm given B-form DNA structure. Thus, altering the spacing between the *tcpA* toxboxes and the  $-35$  box while retaining wild-type spacing between *tcpA* toxboxes was tolerated to a large degree.

Changes in the spacing between the toxboxes at *tcpA* were less well tolerated. A construct having a 1-bp insertion and a construct having a 1-bp deletion between the toxboxes were activated 13- and 26-fold, respectively, and a construct having a 2-bp deletion between the toxboxes was activated 8.3-fold. Any other changes in spacing abrogated activation by ToxT. Therefore, movement of the promoter-distal toxbox relative to the rest of the *tcpA* promoter caused a greater relative defect in activation by ToxT than movement of both toxboxes together.

**Effects of spacing mutations on the *acfA* and *acfD* promoters.** The divergently transcribed *acfA* and *acfD* promoters have a central pair of toxboxes between them that are required for transcription activation of both genes by ToxT (43). However, the spacing of these toxboxes relative to the two core promot-

ers differs (Fig. 1). The *acfD* promoter-proximal toxbox 5' end is located at position  $-46$ , whereas the *acfA* promoter-proximal toxbox 5' end is located at position  $-51$ , a difference of half a turn of the DNA helix. Given this natural difference, we expected to see significant flexibility in the toxbox-promoter spacing with which ToxT could effectively activate transcription of *acfA* and *acfD*. However, this was not the case. The two promoters behaved very similarly when base pairs were inserted between the proximal toxbox and the  $-35$  box (Fig. 2C and 2D). A 1-bp insertion had no effect (*acfD*) or resulted in an even higher level of activation by ToxT (*acfA*). A 2-bp insertion decreased the level of activation of both promoters, and a 3-bp insertion abrogated activation by ToxT. A difference between the *acfA* and *acfD* promoters was observed with the deletion constructs. *acfD* was slightly activated by ToxT (3.5-fold) when 1 bp was deleted between the proximal toxbox and the  $-35$  box, but further deletions abrogated activation. Deletions between the *acfA*-proximal toxbox and the  $-35$  box resulted in high basal transcription levels with no activation conferred by ToxT. Instead, these constructs were slightly repressed by ToxT. *acfA* constructs having additional deletions (4 and 5 bp) were slightly activated by ToxT. It is notable that the *acfA* construct having a 5-bp deletion between the proximal toxbox and the  $-35$  box had the same spacing as the wild-type *acfD* promoter, yet it was very poorly activated by ToxT. In the same vein, a construct having a 5-bp insertion between the *acfD*-proximal toxbox and the  $-35$  box was not activated by ToxT (data not shown). This construct mimicked the spacing at the wild-type *acfA* promoter, yet this spacing was not functional for transcription activation of *acfD* by ToxT.

Changes in spacing between the *acfA* and *acfD* toxboxes were not as well tolerated by ToxT. Both *acfA* and *acfD* constructs having 1-bp insertions between the toxboxes were still activated by ToxT, albeit to a lesser degree for *acfD*. Any larger insertion abrogated activation. Deletion of one or both of the two base pairs between the *acfA* and *acfD* toxboxes resulted in no activation of either gene by ToxT. Thus, as observed for the *tcpA* promoter, changes in spacing between the promoter-proximal toxbox and the core promoter elements had less effect on activation by ToxT than changes in spacing between the toxboxes.

**Effects of spacing mutations on the *tagA* promoter.** ToxT requires a pair of toxboxes in an inverted repeat configuration to activate *tagA* transcription (42). The spacing between the *tagA* inverted repeat toxboxes (8 bp) differs from the spacing between the *acfA* and *acfD* inverted repeat toxboxes (2 bp) (Fig. 1). As we observed for the *tcpA* promoter, ToxT was able to activate transcription of *tagA* constructs having a variety of spacing changes between the promoter-proximal toxbox and the  $-35$  box (Fig. 2E). Constructs having 1- or 2-bp insertions or a 1-bp deletion were activated by ToxT at levels equal to or greater than the level observed with wild-type *tagA* spacing.

Constructs having insertions between the *tagA* toxboxes also exhibited a greater defect in activation by ToxT.  $\beta$ -Galactosidase expression from a construct having a 1-bp insertion and from a construct having a 1-bp deletion between the toxboxes was activated at relatively low levels by ToxT (4.4- and 3.4-fold, respectively). Further changes in spacing between the toxboxes abrogated activation of *tagA* by ToxT. This sensitivity to changes in spacing between the toxboxes exhibited by ToxT at

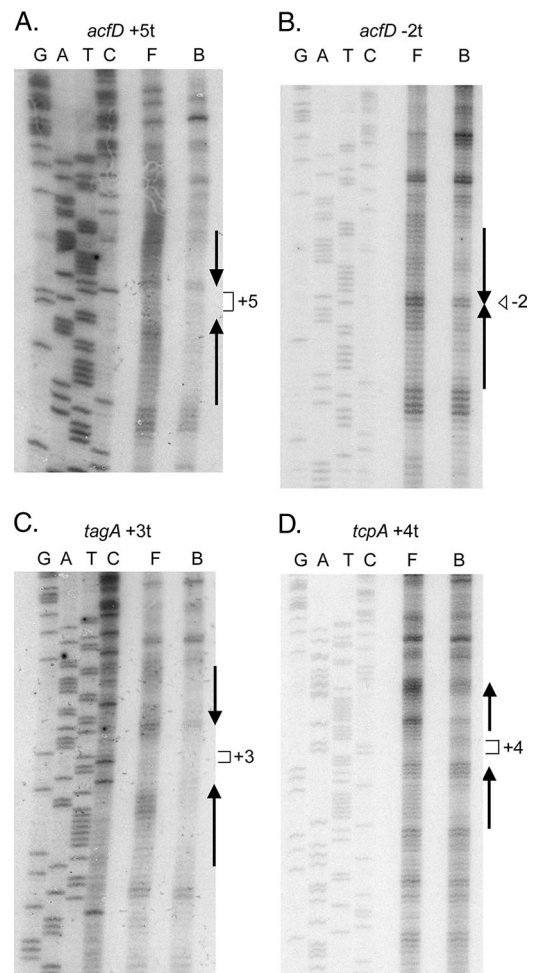


FIG. 3. CP footprinting of ToxT on spacing variants. Experiments were performed using histidine-tagged ToxT and purified end-labeled DNA as described in Materials and Methods. Lanes G, A, T, and C are the sequencing reaction lanes used for size markers, lane F contained free DNA, and lane B contained DNA complexed with ToxT. The numbers indicate the positions of inserted or deleted DNA sequence. (A) CP footprinting for the *acfD* +5t construct. (B) CP footprinting for the *acfD* -2t construct. (C) CP footprinting for the *tagA* +3t construct. (D) CP footprinting for the *tcpA* +4t construct.

*tagA* is similar to what we observed with the *acfA* and *acfD* promoters, suggesting that the inverted repeat toxbox configuration, regardless of the wild-type spacing between toxboxes, is not as tolerant of spacing changes as the direct repeat configuration at *tcpA*.

**CP footprinting of ToxT on spacing variants.** To examine the ToxT occupancy of toxboxes with changes in the spacing between them, CP footprinting was performed. Previously, we found that 5- or 10-bp insertions between direct repeat or inverted repeat toxboxes did not affect the footprinting pattern of ToxT, although these insertions abrogated transcription activation by ToxT (41, 43). As shown in Fig. 3, we footprinted ToxT on the *acfD* +5t, *acfD* -2t, *tcpA* +4t, and *tagA* +3t fusion constructs. All of these constructs exhibited defects in ToxT activation (Fig. 2). The CP footprinting data obtained for the *acfD* +5t, *tcpA* +4t, and *tagA* +3t constructs indicate that in each case ToxT occupied both toxboxes regardless of their

position relative to each other, as we previously observed with the *acfD* and *tcpA* promoters (41, 43). These results suggest that rotating the toxboxes by insertional mutation does not disrupt ToxT binding and also suggest that there is independent binding of ToxT to individual toxboxes. The CP footprinting data obtained for the *acfD* -2t construct revealed a different pattern of binding (Fig. 3). The *acfD*-distal toxbox was clearly protected by ToxT, whereas the *acfD*-proximal toxbox was more weakly protected by ToxT. Because the *acfD* -2t construct eliminated the only two base pairs between the toxboxes, it is likely that ToxT monomers impeded binding of each other on this construct through steric hindrance. In this case the observed defect in ToxT-activated transcription was likely due to the absence of ToxT bound to the *acfD*-proximal promoter.

### DISCUSSION

The experiments described in this paper examined the ability of ToxT to activate transcription of *V. cholerae* virulence gene promoters having altered spacing between the toxboxes and the core promoter elements. Our results indicate that different toxbox configurations have different spacing requirements for ToxT activity. This finding suggests that the interactions between ToxT and RNAP involved in transcription activation differ depending on the toxbox configuration. All known ToxT-activated promoters have a class I architecture, suggesting that ToxT interacts with the  $\alpha$  C-terminal domain ( $\alpha$ -CTD) of RNAP (2). Overexpression of a mutant  $\alpha$  subunit lacking the CTD resulted in a loss of *tcpA* activation by ToxT, strongly suggesting that this interaction is essential for transcription activation (19).

The promoter-proximal toxboxes at the *acfD*, *tcpA* and *tagA* promoters are located at similar positions and in the same orientation relative to the core promoter, and the differences between these promoters are the orientation of the promoter-distal toxbox and the spacing between the toxboxes (Fig. 1). ToxT was able to activate constructs from all three promoters having significant changes in spacing between the promoter-proximal toxbox and the -35 box. One explanation for this similarity is that the interactions between the ToxT monomer bound to the promoter-proximal toxbox and the RNAP  $\alpha$ -CTD are similar at these three promoters. A caveat is that insertions or deletions between the core promoter and the proximal toxbox alter the spacing of both toxboxes relative to the promoter, so such changes could potentially affect interactions between both ToxT monomers bound to the two toxboxes and both  $\alpha$ -CTD subunits. Further similarity between the *acfD*, *tcpA*, and *tagA* promoters was found in previous work with the ToxT antagonist virstatin (20), which interacts with the ToxT N-terminal domain and may inhibit interaction between monomers (37). These three promoters were found to be the promoters most sensitive to treatment with virstatin.

Altering the spacing between the toxboxes had different effects on the inverted repeat and direct repeat promoters. Transcription activation by ToxT from the inverted repeat toxbox pairs at *acfD* and *tagA* was severely reduced by a 1-bp insertion or deletion between the toxboxes, and a 2-bp insertion or deletion resulted in a complete loss of activation by ToxT. At the *acfA* inverted repeat toxbox pair, ToxT was able

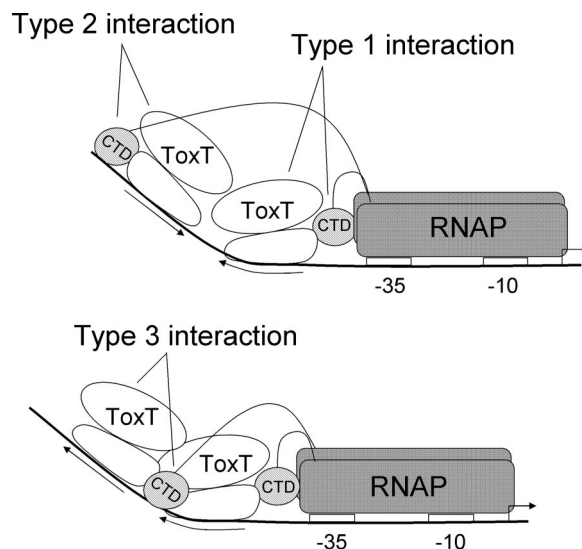


FIG. 4. Model showing three types of interactions between ToxT and RNAP in transcription activation at promoters having different toxbox configurations. The open ellipses represent ToxT, the arrows represent toxboxes, and the shaded ellipses represent the RNAP  $\alpha$ -CTDs. -35 and -10 boxes are represented by open rectangles, and the transcription start site is indicated by a bent arrow.

to activate a promoter having a 1-bp insertion between the toxboxes, but any other changes resulted in a loss of activation. Thus, the inverted repeat promoters are very sensitive to altered spacing between toxboxes. If ToxT monomers dimerize and this interaction is required for transcription activation, insertions between toxboxes could interfere; these changes in spacing could also interfere with interactions between ToxT bound to the distal toxbox and the RNAP  $\alpha$ -CTD. Changes in spacing between the direct repeat toxbox pair at *tcpA* had less severe effects. ToxT activated transcription from constructs having a 1-bp insertion and a 1-bp deletion between the toxboxes 13- and 26-fold, respectively, and activated a construct having a 2-bp deletion 8.3-fold. This difference in sensitivity to changes in spacing between the inverted repeat and direct repeat promoters suggests that the interactions between ToxT bound to the promoter-distal toxbox and RNAP are similar at the *acfA*, *acfD*, and *tagA* promoters and different at the *tcpA* promoter, as we have previously proposed (41).

The promoter that has the lowest amplitude of activation by ToxT, *aldA*, was also the promoter most sensitive to any spacing changes. The *aldA* promoter is the only known promoter at which ToxT requires a single binding site for activation, and any change in spacing between this toxbox and the -35 box eliminated activation by ToxT. Spacing changes upstream of this toxbox had no effect, confirming that ToxT requires only this single site for activation of *aldA*. In addition to the presence of a single toxbox, the orientation of the *aldA* toxbox is opposite that of other promoter-proximal toxboxes. This suggests that the interactions between ToxT and RNAP involved in activation of *aldA* are different than those at other promoters having a toxbox at a similar position.

Based on the results described above, we propose that ToxT interacts with RNAP in at least three different ways based on the position and orientation of the toxboxes and that these

interactions have different spacing requirements (Fig. 4). A type 1 interaction occurs between the RNAP  $\alpha$ -CTD and ToxT at a promoter-proximal toxbox oriented so that it "points away" from the promoter. This is the case for *tcpA*, *tagA*, and *acfD*. The type 1 interaction is relatively spacing insensitive and may involve an interaction between  $\alpha$ -CTD and ToxT that is not localized to the DNA, as has been observed with some other AraC family members (4, 36). The type 1 interaction is very virustatin sensitive. A type 2 interaction between  $\alpha$ -CTD and ToxT occurs at toxboxes oriented so that they "point toward" the promoter. This is the case for the inverted repeat toxbox promoters *acfA*, *acfD*, and *tagA*, as well as the single toxbox promoter *aldA*. The type 2 interaction is very spacing sensitive, as a difference of even 1 bp from the wild-type spacing abrogates transcription activation at some promoters. However, the *acfA* and *aldA* promoters were the promoters least sensitive to virustatin treatment (37), suggesting that the type 2 interaction is virustatin insensitive. A type 3 interaction occurs between  $\alpha$ -CTD and ToxT at a distal toxbox oriented so that it "points away" from the promoter. This occurs at the *tcpA* promoter and has intermediate spacing sensitivity, as insertion or deletion of 1 bp is tolerated relatively well but larger changes are not well tolerated. In terms of activation by ToxT, the *tcpA* promoter, having type 1 and type 3 interactions, is most highly activated, the three promoters having type 1 and type 2 interactions are activated at intermediate levels, and the *aldA* promoter having only a type 2 interaction is weakly activated.

One promoter that does not fit as well into this simple scheme is *acfA*. *acfA* has a promoter-proximal toxbox that is located approximately 5 bp further upstream than is typical for other ToxT-activated promoters, and spacing alteration affects it differently than it affects the other promoters. Surprisingly, changing the spacing between the proximal *acfA* toxbox and the  $-35$  box to resemble the spacing for more typical promoters did not result in a construct that was activated well by ToxT. This was despite the fact that the same toxbox pair is used by ToxT to activate *acfD* transcription with the more typical spacing. Previously, we observed that mutations in the toxbox pair between *acfA* and *acfD* had very similar effects on transcription activation of both promoters by ToxT (43). The results described here suggest that the DNA sequences of the individual toxboxes may play a further role in activation. Mutagenesis of every base pair within the *tcpA* toxboxes identified 7 of 13 bp that were important for activation of this promoter (41), but there may be additional sequence requirements for toxboxes at promoters having different configurations, such as *acfA*.

Much previous work in many laboratories on spacing requirements of class I promoters has focused on the effects of inserting or deleting a half or full turn of the DNA helix, although a few studies similar to this study have been described previously. The most relevant example is the MarA protein, another member of the AraC/XylS family that binds to DNA as a monomer. MarA is able to activate transcription from both class I and class II promoters depending on the location and orientation of its binding sites (27). Studies on the spacing between a MarA binding site and the  $-35$  box required for transcription activation of a class I promoter found that the wild-type spacing (16 bp) is optimal and that there was a gradual decrease in activation amplitude as base pairs were

either inserted or deleted to alter the spacing (28). Interestingly, the observed spacing range with which MarA was able to activate transcription is similar to the range for the ToxT-activated promoters described here as having a type 1 interaction with RNAP  $\alpha$ -CTD. SoxS, a transcription activator closely related to MarA that recognizes the same DNA binding site, was found to require one particular orientation of its binding site when located close to the promoter and the opposite orientation of its binding site when located further upstream (44). This finding clearly differs from our observation that ToxT can use binding sites in either orientation at similar locations relative to the core promoter. A study of *Escherichia coli* CytR, a repressor in the LacI family, showed that CytR was able to function as a repressor when its individual binding sites were separated by between 1 and 16 bp in 2-bp increments (21). CytR binds to DNA as a dimer, and differences in the affinity of CytR for its binding sites correlated with the degree of repression observed with various spacings. We have previously observed that ToxT binds to DNA as a monomer and that rotating its binding sites by even a half turn of the DNA did not affect their occupancy by ToxT (41, 43).

In summary, we analyzed the promoter spacing requirements for *V. cholerae* ToxT to activate transcription of virulence genes. Our results indicate that different interactions are likely to occur between ToxT and RNAP depending on the position and orientation of ToxT binding sites relative to each other and relative to the  $-35$  box and that these ToxT-RNAP interactions differ in their spacing flexibility.

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