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The toxbox: specific DNA sequence requirements for activation of *Vibrio cholerae* virulence genes by ToxT

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Summary

The Gram-negative, curved rod *Vibrio cholerae* causes the severe diarrhoeal disease cholera. The two major virulence factors produced by *V. cholerae* during infection are the cholera toxin (CT) and the toxin-coregulated pilus (TCP). Transcription of the genes encoding both CT and the components of the TCP is directly activated by ToxT, a transcription factor in the AraC/XylS family. ToxT binds upstream of the *ctxAB* genes, encoding CT, and upstream of *tcpA*, the first gene in a large operon encoding the components of the TCP. The DNA sequences upstream of *ctxAB* and *tcpA* that contain ToxT binding sites do not have any significant similarity other than being AT-rich. Extensive site-directed mutagenesis was performed on the region upstream of *tcpA* previously shown to be protected by ToxT, and we identified specific base pairs important for activation of *tcpA* transcription by ToxT. This genetic approach was complemented by copper-phenanthroline footprinting experiments that showed protection by ToxT of the base pairs identified as most important for transcription activation in the mutagenesis experiments. Based on this new information and on previous work, we propose the presence of a ToxT-binding motif – the 'toxbox' – in promoters regulated by ToxT. At *tcpA*, two toxbox elements are present in a direct repeat configuration and both are required for activation of transcription by ToxT. The identity of only a few of the base pairs within the toxbox is important for activation by ToxT, and we term these the core toxbox elements. Lastly, we examined ToxT binding to a mutant having 5 bp inserted between the two toxboxes at *tcpA* and found that occupancy of both binding sites is retained regardless of the positions of the binding sites relative to each other on the face of the DNA. This suggests that ToxT binds independently as a monomer to each toxbox in the *tcpA* direct repeat, in accordance with what we observed previously with the inverted repeat ToxT sites between *acfA* and *acfD*.

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Introduction

Vibrio cholerae secretes cholera toxin (CT), which is responsible for the clinical aspects of cholera disease. The other major virulence factor produced by *V. cholerae* is the toxin-coregulated pilus (TCP), a type IV bundle-forming pilus required for intestinal colonization by *V. cholerae* (Taylor *et al*., 1987; Herrington *et al*., 1988; Attridge *et al*., 1996; Thelin and Taylor, 1996). Expression of the *V. cholerae* virulence genes is regulated by a cascade of transcription factors. The virulence genes are collectively known as the ToxR regulon for historical purposes, because ToxR was the first regulator of virulence that was identified. However, it is ultimately ToxT protein that directly activates the vast majority of *V. cholerae* virulence genes, including *ctxAB* and *tcpA*.

ToxT, a 32 kDa, 276-amino-acid protein, is a member of the large AraC/XylS family of transcription regulators (Higgins *et al*., 1992). This family shares a domain of approximately 100 amino acids that contains two helix–turn–helix DNA binding motifs (Gallegos *et al*., 1997; Martin and Rosner, 2001; Tobes and Ramos, 2002). In ToxT, the AraC/XylS family domain consists of the 104 C-terminal amino acids. The remaining 172 N-terminal amino acids of ToxT, which presumably form a separate N-terminal domain (NTD), have unknown function and BLAST searches of the protein database with this region alone yield no homology to any other protein. Environmental strains of *V. cholerae* exhibit significant variation in this region of ToxT, whereas the C-terminal AraC/XylS region of ToxT is nearly invariant (Mukhopadhyay *et al*., 2001). Common functions for secondary domains among other AraC/XylS family members include effector binding and multimerization. ToxT is not known to bind any effectors, although bile may be a negative regulator of its activity (Schuhmacher and Klose, 1999). Recently we have found evidence that ToxT binds independently, most likely as monomers, to its two binding sites between *acfA* and *acfD*, which encode components of the accessory colonization factor (Withey and DiRita, 2005a). ToxT activates transcription of both *acfA* and *acfD* from this central position and requires both binding sites for activation of each

gene. The two *acfA* and *acfD* ToxT binding sites are configured as an inverted repeat separated by 2 bp.

Our previous study of ToxT binding sites between *acfA* and *acfD* proposed a consensus DNA binding sequence for ToxT consisting of a 13 bp site with a tract of T nucleotides near the 5′ end of the site and A/T-rich sequence near the 3′ end of the site (Withey and DiRita, 2005a). Tracts of four or more consecutive T or A mononucleotides are the only common features found upstream of all genes known to be activated by ToxT, and T tracts are the only obvious conserved sequences found among ToxT binding sites (Withey and DiRita, 2005a,b). Prior work on the role of ToxT in activation of *tcpA* and *ctxAB* located the regions to which ToxT binds upstream of these genes using genetic techniques and DNase I footprinting, and found that both ToxT binding regions contain multiple poly-T tracts (Hulbert and Taylor, 2002; Yu and DiRita, 2002). However, little information about specific sequence requirements for ToxT binding and activation was gleaned from these studies.

In this report, we investigate in greater detail the DNA sequence requirements for ToxT binding and activation of *tcpA*. By measuring β-galactosidase levels produced from *tcpA::lacZ* constructs having mutations in the ToxT binding sequence in *V. cholerae* strains having either wild-type *toxT* or a *toxT* deletion, we determined which specific base pairs within the ToxT-binding region are most important for activation of *tcpA* transcription. These experiments were complemented by copper-phenanthroline footprinting experiments. We find that ToxT uses two 13 bp binding sites in a direct repeat configuration to activate *tcpA* transcription; we propose that the ToxT binding site be named the 'toxbox'. Our results further indicate that the identities of only a few of the base pairs within each of the 13 bp toxboxes are essential for *tcpA* activation. Finally, results from constructs having 5 bp and 10 bp inserted between the two ToxT binding sites suggest that ToxT binds independently to the two direct repeat *tcpA* toxboxes, likely as monomers. However, appropriate spacing of the bound ToxT molecules relative to the promoter is critical for transcription activation. These findings are similar to those we made previously with the inverted repeat ToxT sites between *acfA* and *acfD* (Withey and DiRita, 2005a).

Results

Rough mapping of the ToxT binding sites using double point mutations

We began our studies of the DNA sequence requirements for ToxT-directed transcription of *tcpA* by making pairs of point mutations in the region upstream of *tcpA* and assessing the ability of ToxT to activate transcription from plasmids carrying the mutated sequences fused to *lacZ*. Previous work identified a region between approximately −42 and −84 relative to the start of *tcpA* transcription as required for ToxT-directed transcription of *tcpA* (Fig. 1) (Hulbert and Taylor, 2002; Yu and DiRita, 2002), and this region was also protected by ToxT in DNase I footprinting experiments (Yu and DiRita, 2002). The notable features of the DNA sequence in this region are three tracts of consecutive A/T nucleotides. We will refer here to these 'T tracts' on the template strand instead of the corresponding 'A tracts' on the non-template strand for the sake of consistency with our earlier studies (Withey and DiRita, 2005a,b). Two of the tracts contain seven T nucleotides, and the third contains six T nucleotides (Figs 1 and 2). Previous work indicated that mutations to the first (promoter-proximal) and third (promoter-distal) T tracts caused defects in ToxT-directed *tcpA* transcription (Hulbert and Taylor, 2002). However, the majority of these mutations were single base pair deletions, which effectively alter all of the sequence upstream of the mutation and thus confer little information about specific sequence requirements.

Twelve constructs having pairs of mutations in the region protected by ToxT in DNase I footprinting experiments (Yu and DiRita, 2002) were created (Fig. S1). The

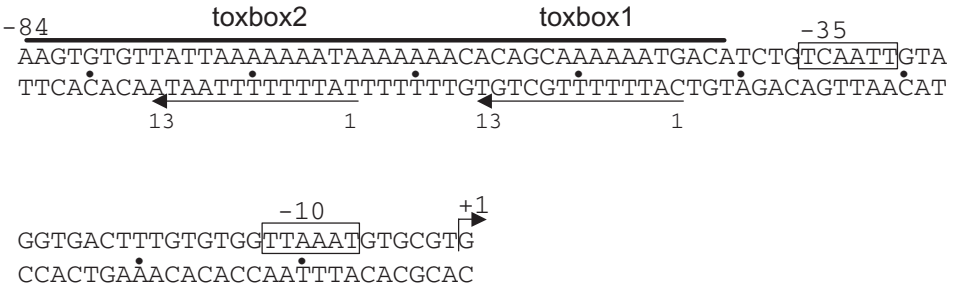
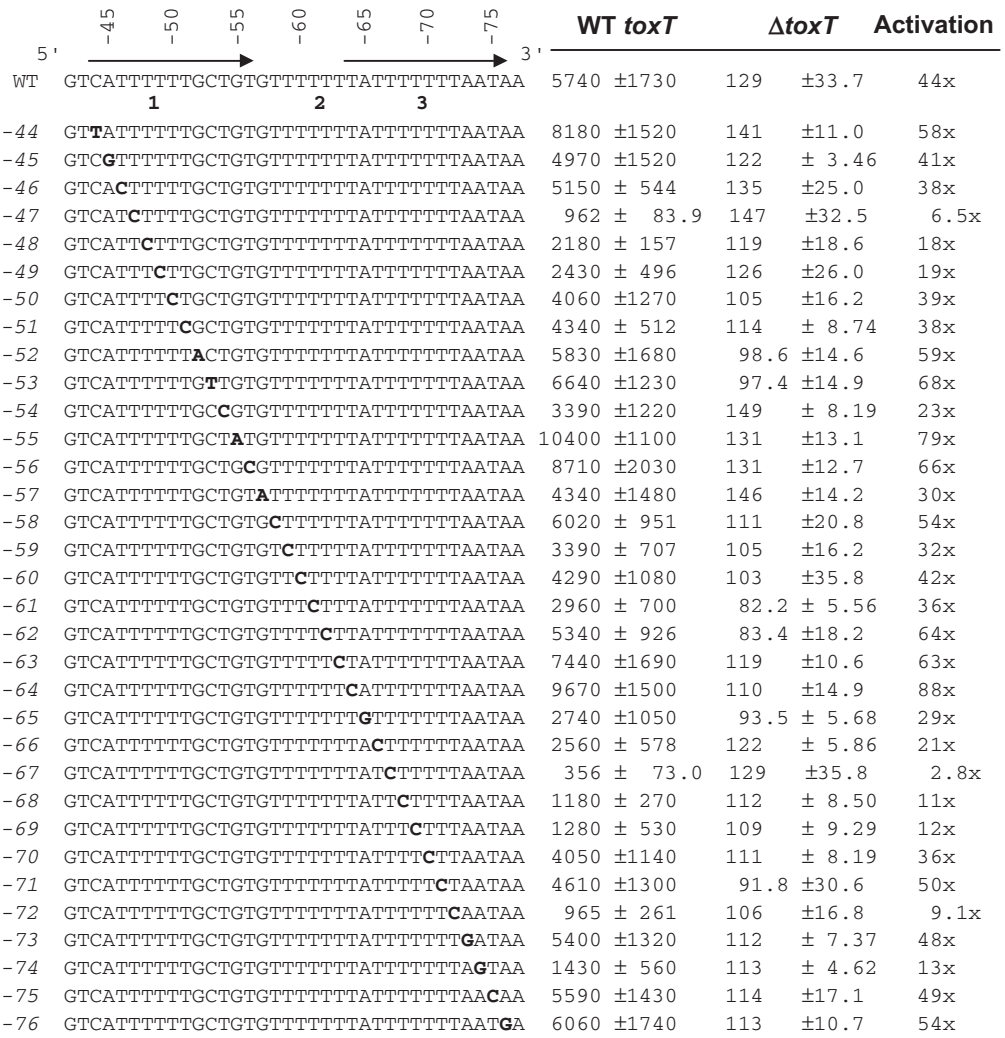


Fig. 1. Sequence of the region upstream of *tcpA* required for ToxT-directed transcription. The sequence protected by ToxT in DNase I footprinting experiments (Yu and DiRita, 2002) is indicated by the heavy line over the sequence. The putative core −10 and −35 promoter elements are indicated by boxes. The two ToxT binding sites required for activation of *tcpA* are indicated by arrows below the sequence and designated toxbox1 and toxbox2, for the promoter-proximal and promoter-distal sites, respectively, above the sequence. The numbering below the binding sites indicates the numerical toxbox positions from 1 to 13 referred to in the text.



		WT *toxT*	Δ*toxT*	Activation
WT	GTCATTTTTTGCTGTGTTTTTTTATTTTTTTAATAA	5740 ±1730	129 ±33.7	44x
-44	GTTATTTTTTGCTGTGTTTTTTTATTTTTTTAATAA	8180 ±1520	141 ±11.0	58x
-45	GTCGTTTTTTGCTGTGTTTTTTTATTTTTTTAATAA	4970 ±1520	122 ± 3.46	41x
-46	GTCACTTTTTGCTGTGTTTTTTTATTTTTTTAATAA	5150 ± 544	135 ±25.0	38x
-47	GTCATCTTTTGCTGTGTTTTTTTATTTTTTTAATAA	962 ± 83.9	147 ±32.5	6.5x
-48	GTCATTCTTTGCTGTGTTTTTTTATTTTTTTAATAA	2180 ± 157	119 ±18.6	18x
-49	GTCATTTCTTGCTGTGTTTTTTTATTTTTTTAATAA	2430 ± 496	126 ±26.0	19x
-50	GTCATTTTCTGCTGTGTTTTTTTATTTTTTTAATAA	4060 ±1270	105 ±16.2	39x
-51	GTCATTTTTCGCTGTGTTTTTTTATTTTTTTAATAA	4340 ± 512	114 ± 8.74	38x
-52	GTCATTTTTTACTGTGTTTTTTTATTTTTTTAATAA	5830 ±1680	98.6 ±14.6	59x
-53	GTCATTTTTTGTTGTGTTTTTTTATTTTTTTAATAA	6640 ±1230	97.4 ±14.9	68x
-54	GTCATTTTTTGCCGTGTTTTTTTATTTTTTTAATAA	3390 ±1220	149 ± 8.19	23x
-55	GTCATTTTTTGCTATGTTTTTTTATTTTTTTAATAA	10400 ±1100	131 ±13.1	79x
-56	GTCATTTTTTGCTGCGTTTTTTTATTTTTTTAATAA	8710 ±2030	131 ±12.7	66x
-57	GTCATTTTTTGCTGTATTTTTTTATTTTTTTAATAA	4340 ±1480	146 ±14.2	30x
-58	GTCATTTTTTGCTGTGCTTTTTTATTTTTTTAATAA	6020 ± 951	111 ±20.8	54x
-59	GTCATTTTTTGCTGTGTCTTTTTATTTTTTTAATAA	3390 ± 707	105 ±16.2	32x
-60	GTCATTTTTTGCTGTGTTCTTTTATTTTTTTAATAA	4290 ±1080	103 ±35.8	42x
-61	GTCATTTTTTGCTGTGTTTCTTTATTTTTTTAATAA	2960 ± 700	82.2 ± 5.56	36x
-62	GTCATTTTTTGCTGTGTTTTCTTATTTTTTTAATAA	5340 ± 926	83.4 ±18.2	64x
-63	GTCATTTTTTGCTGTGTTTTTCTATTTTTTTAATAA	7440 ±1690	119 ±10.6	63x
-64	GTCATTTTTTGCTGTGTTTTTTCATTTTTTTAATAA	9670 ±1500	110 ±14.9	88x
-65	GTCATTTTTTGCTGTGTTTTTTTGTTTTTTTAATAA	2740 ±1050	93.5 ± 5.68	29x
-66	GTCATTTTTTGCTGTGTTTTTTTACTTTTTTAATAA	2560 ± 578	122 ± 5.86	21x
-67	GTCATTTTTTGCTGTGTTTTTTTATCTTTTTAATAA	356 ± 73.0	129 ±35.8	2.8x
-68	GTCATTTTTTGCTGTGTTTTTTTATTCTTTTAATAA	1180 ± 270	112 ± 8.50	11x
-69	GTCATTTTTTGCTGTGTTTTTTTATTTCTTTAATAA	1280 ± 530	109 ± 9.29	12x
-70	GTCATTTTTTGCTGTGTTTTTTTATTTTCTTAATAA	4050 ±1140	111 ± 8.19	36x
-71	GTCATTTTTTGCTGTGTTTTTTTATTTTTCTAATAA	4610 ±1300	91.8 ±30.6	50x
-72	GTCATTTTTTGCTGTGTTTTTTTATTTTTTCAATAA	965 ± 261	106 ±16.8	9.1x
-73	GTCATTTTTTGCTGTGTTTTTTTATTTTTTTGATAA	5400 ±1320	112 ± 7.37	48x
-74	GTCATTTTTTGCTGTGTTTTTTTATTTTTTTAGTAA	1430 ± 560	113 ± 4.62	13x
-75	GTCATTTTTTGCTGTGTTTTTTTATTTTTTTAACAA	5590 ±1430	114 ±17.1	49x
-76	GTCATTTTTTGCTGTGTTTTTTTATTTTTTTAATGA	6060 ±1740	113 ±10.7	54x

Fig. 2. Single point mutations to the *tcpA* ToxT binding region. The mutated nucleotides are shown in bold. Only the template strand sequence (lower strand in Fig. 1) is shown. The results of β-galactosidase assays in wild-type and Δ*toxT* strains carrying plasmids with the indicated mutations upstream of the *tcpA::lacZ* fusion are shown to the right of the sequences. 'Activation' indicates the fold-difference in β-galactosidase activity between the wild-type *toxT* and Δ*toxT* strains for that construct. A minimum of three separate experiments were performed for each strain, and the values shown are the mean ± the standard deviation.

mutant sequences were cloned in plasmid pTL61T, which carries a promoterless *lacZ* gene downstream from multiple restriction sites. The ability of ToxT to activate transcription of these *tcpA::lacZ* constructs was assessed by measuring β-galactosidase activity in *V. cholerae* strains having either wild-type *toxT* or a *toxT* deletion. The double point mutations having the most severe effect on ToxT-directed *tcpA* transcription were located in the first and third T tracts (Fig. S1). Both the *tcpA–47–49* and *tcpA–68–70* mutations to these two T tracts caused a reduction in the level of induction by ToxT of *tcpA* transcription from 44-fold, as observed in the wild-type *tcpA::lacZ* fusion, to under twofold. Mutations to the second (centre) T tract had a less dramatic effect. The *tcpA-60–62* construct, which had the largest defect in ToxT-directed transcription within the second T tract, still exhibited 14-fold induction by ToxT of *tcpA* transcription. This suggests that the second T tract may play a structural role in ToxT-directed transcription of *tcpA*, rather than being directly contacted by ToxT.

These results are consistent with those from a previous study that used a genetic screen to identify mutant sequences from which ToxT was unable to activate *tcpA* (Hulbert and Taylor, 2002). In that study no mutations were identified within the second T tract. These results further suggest that there are two ToxT binding sites located upstream of *tcpA*, each containing one T tract, and that both of the binding sites are required for ToxT-directed transcription of *tcpA*. This arrangement is similar to the ToxT binding sites we identified previously (Withey and DiRita, 2005a,b) with one significant difference: in the

case of *tcpA*, both T tracts are located on the same DNA strand, suggesting that the *tcpA* ToxT binding sites are in a direct repeat configuration, in contrast to the ToxT binding sites in inverted repeat configurations we observed between *acfA* and *acfD* (Withey and DiRita, 2005a) and upstream of *tagA* (Withey and DiRita, 2005b), or the single ToxT binding site we identified upstream of *aldA* (Withey and DiRita, 2005b).

Mutagenesis of the entire ToxT-binding region upstream of tcpA

To gain further insight into the DNA sequence requirements for ToxT-directed transcription of *tcpA*, we constructed a plasmid collection of single base pair *tcpA::lacZ* mutants encompassing the entire region between –44 and –76 relative to the start of *tcpA* transcription. As shown in Fig. 2, all of the mutations changed an A/T base pair to a G/C base pair or vice versa. β-Galactosidase levels were measured in *V. cholerae* carrying these plasmids and having either wild-type *toxT* or a *toxT* deletion (Fig. 2). The β-galactosidase results from the strains having wild-type *toxT* are shown graphically in Fig. 3 above each nucleotide. It is evident from these experiments that only a small number of single base pair mutations significantly affect the ability of ToxT to activate *tcpA* transcription. Calculations of statistical significance by Student's *t*-test, in this case meaning $P < 0.03$, indicated that mutations at 10 positions caused a significant loss of activation by ToxT (asterisks in Fig. 3). Mutations to the first and third T tracts at positions –47 to –49 and –67 to –69 dramatically reduced the ability of ToxT to activate *tcpA* transcription. Mutations to positions –72 and –74 also severely reduced ToxT-directed *tcpA* transcription. Mutations to positions –65 and –66 caused a significant decrease in ToxT-directed *tcpA* transcription. For all of the above mutations $P < 0.015$. Mutations within the second T tract had little to no effect on ToxT-directed *tcpA* transcription with the exception of positions –59 and –61; these mutations caused a moderate decrease in ToxT-directed *tcpA* transcription, although only the β-galactosidase levels from the –61 mutation are outside of the margin of error, and then only slightly. *P*-values for positions –59 and –61 were 0.058 and 0.031 respectively. Finally, mutation to position –54 produced a decrease in ToxT-directed transcription similar to those produced by the mutations to positions –59 and –61, but again this decrease was within the margin of error and a double mutation of positions –54 and –55 was not at all reduced for ToxT-directed transcription (Fig. S1), suggesting that the identity of position –54 has little effect on activation by ToxT; for this mutation, $P = 0.068$.

These results, together with our studies of other ToxT binding sites (Withey and DiRita, 2005a,b), which indicated that ToxT binds to a 13 bp site and that all ToxT binding sites contain a conserved T tract near the 5′ end, suggest that ToxT binds to two sites in a direct repeat configuration upstream of *tcpA*. However, there is a clear difference between the two binding sites in terms of the sequence requirements for activation of *tcpA* by ToxT. Both the promoter-proximal and promoter-distal sites, which we will refer to as the toxbox1 and toxbox2 sites, respectively, share a requirement for three consecutive T nucleotides at positions 4–6 from the 5′ end of the binding sites, whereas toxbox2 has additional sequence requirements at positions 2, 3, 9 and 11 (Figs 1 and 3).

Copper-phenanthroline footprinting of ToxT on the tcpA *promoter region*

Previously, DNase I footprinting was used to localize the region of ToxT binding upstream of *tcpA* (Yu and DiRita, 2002). To get a more refined picture of ToxT binding

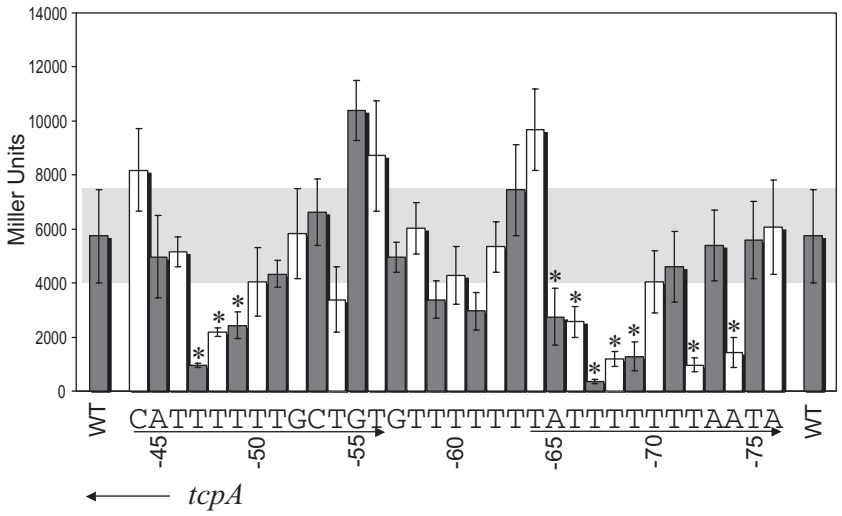


Fig. 3. Graphical representation of the β-galactosidase results from *tcpA::lacZ* constructs having double point mutations. Results from strains having the wild-type (WT) *tcpA::lacZ* fusion are shown at both sides of the figure, and the grey box across the figure indicates the standard deviation from the mean for the wild-type *tcpA::lacZ* fusion. The values shown are only those measured in wild-type ToxT *V. cholerae*. Bars are located over the respective nucleotide that is mutated in each construct. Asterisks indicate positions to which mutation caused a statistically significant decrease in β-galactosidase levels according to Student's *t*-test ($P < 0.03$).

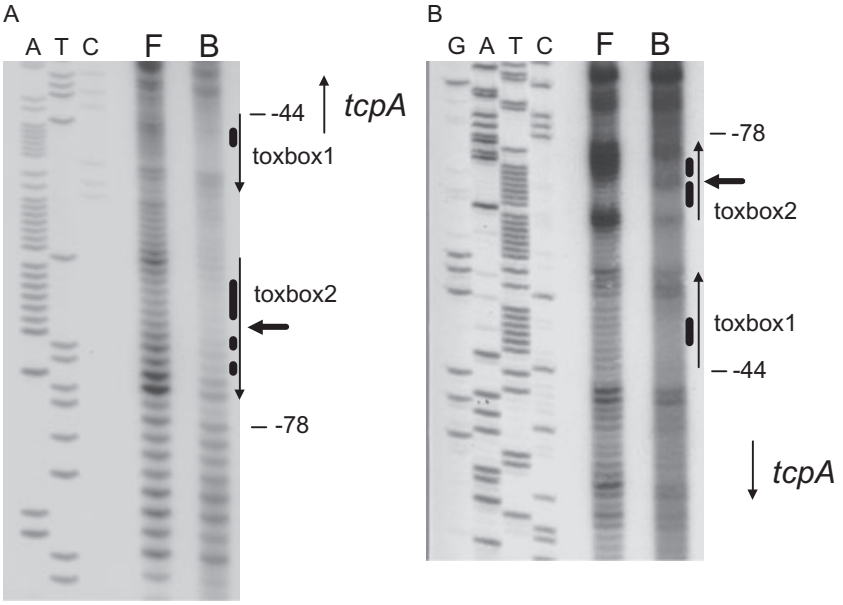


Fig. 4. Copper-phenanthroline footprints of ToxT on *tcpA*. ToxT binding sites toxbox1 and toxbox2 are indicated by arrows. The black bars denote the locations of the core toxbox determinants. Numbers refer to positions relative to the start of *tcpA* transcription. F = free DNA, B = ToxT/DNA complex. Heavy arrows indicate the positions of a hypersensitive site found only on the template strand.
A. Non-template strand. The letters 'A, T, C' above the left side of the figure refer to the respective lanes of the sequencing ladder.
B. Template strand. The letters 'G, A, T, C' above the left side of the figure refer to the respective lanes of the sequencing ladder.

at *tcpA*, here we have instead used copper-1,10-phenanthroline as the DNA cleavage agent for footprinting. This technique has higher resolution as copper-1,10-phenanthroline has much less sequence cleavage specificity than DNase I and cleaves at essentially every nucleotide (Papavassiliou, 1994; Withey and DiRita, 2005a,b). Purified ToxT bearing six histidine residues at its N-terminus (H_6-ToxT) was used in the footprinting experiments; previous work demonstrated that H_6-ToxT is functional both *in vivo* and *in vitro* for transcription activation (Yu and DiRita, 2002; Withey and DiRita, 2005a,b).

Copper-phenanthroline footprinting of H_6-ToxT on wild-type *tcpA* DNA exhibited protection, as expected, within the two T tracts shown to be essential for ToxT-directed *tcpA* transcription (Fig. 4A and B). Some protection was also conferred by H_6-ToxT to the surrounding base pairs that encompass the ToxT binding sites, especially toxbox2. However, no significant protection was conferred by H_6-ToxT to nucleotides 9–13 of toxbox1. These results are consistent with the results described above indicating the specific sequence requirements for activation of *tcpA* by ToxT, and with the sequence differences observed between the toxbox1 and toxbox2 sites. As predicted, much less protection was observed between the two ToxT binding sites, again suggesting that the central T tract does not play a direct role in ToxT binding and supporting our designated location for the two ToxT binding sites. In Fig. 4B (template strand) the band intensity throughout the bound complex lane is somewhat lower than in the free DNA lane; however, there is still significant protection evident within the areas of the binding sites. Furthermore, there is a difference in protection when comparing toxbox2 in the template (Fig. 4B) versus the non-template (Fig. 4A) strands; there is an unprotected or even hypersensitive area present at positions 7 and 8 (Fig. 1) of toxbox2 in the template strand footprint that is not present in the non-template strand footprint (heavy arrows in Fig. 4A and B). However, this lack of protection is consistent with our finding that mutations at these positions do not affect activation of *tcpA* by ToxT, and suggests that ToxT may bend the DNA at this position.

Alteration of the spacing between the ToxT binding sites at tcpA

Our previous work has indicated that ToxT activates transcription of *acfA*, *acfD* and *tagA* using binding sites in inverted repeat configurations (Withey and DiRita, 2005a,b), and that only a single ToxT site exists upstream of *aldA* (Withey and DiRita, 2005b). Furthermore, we found evidence that ToxT binds as independent monomers to its two sites between *acfA* and *acfD* and that appropriate spacing between the binding sites is important for activation of both genes by ToxT (Withey and DiRita, 2005a). Because the experiments described here suggest that ToxT activates transcription of *tcpA* from two binding sites in a direct repeat configuration, in contrast to our previous studies of other genes within the ToxT regulon, we investigated whether ToxT would activate *tcpA* transcription if 5 bp or 10 bp were inserted between its two binding sites. This is especially germane given the recent finding that the NTD of ToxT may have dimerization activity (Prouty *et al*., 2005). The 5 bp insertion would rotate toxbox1 and toxbox2 approximately one half-turn of the DNA helix relative to each other, which would almost certainly disrupt any interactions between ToxT molecules

Table 1. Effects of insertions between the *tcpA* toxboxes on activation by ToxT.

Construct	Wild-type *toxT* (Miller units)	Δ*toxT* (Miller units)	Fold-activation
tcpA wild-type	5740 ± 1730	120 ± 35.5	48×
tcpA +5	92 ± 10.4	83.3 ± 1.47	1.1×
tcpA +10	86.4 ± 9.83	88.9 ± 9.76	0.97×

bound to the two sites, and the 10 bp insertion would rotate toxbox1 and toxbox2 approximately one full turn of the DNA helix relative to each other. The insertions could also affect potential interactions between ToxT bound to toxbox2 and the C-terminal domain of the α-subunit of RNA polymerase (RNAP) (α-CTD); previous work indicated that ToxT most likely interacts with the α-CTD (Hulbert and Taylor, 2002).

We assessed the ability of ToxT to activate transcription from the abnormally spaced binding sites using *tcpA*::*lacZ* fusions as described earlier. As shown in Table 1, both the 5 bp and 10 bp insertions between toxbox1 and toxbox2 completely abrogate activation of *tcpA* by ToxT. For the 5 bp insertion, the loss of activation could be due to either the lack of a necessary interaction between ToxT molecules, resulting in a loss of DNA binding by ToxT, or to loss of an interaction between ToxT bound to toxbox2, which is now on the opposite face of the DNA relative to the promoter, and α-CTD. To distinguish between the two possibilities, we used copper-phenanthroline footprinting to determine the occupancy by H_6-ToxT of toxbox1 and toxbox2 sites having the 5 bp spacing mutation. As shown in Fig. 5, both ToxT binding sites are protected regardless of the 5 bp insertion between them, suggesting that ToxT binds independently to the toxbox1 and toxbox2 sites, and that loss of interaction between ToxT bound to toxbox2 and the α-CTD is responsible for the defect in transcription activation conferred by the 5 bp insertion.

Discussion

In this report we present experiments designed to identify specific DNA sequence requirements for transcription activation of the *tcpA* operon by ToxT. The *tcpA* operon includes the genes encoding components of the TCP and the *toxT* gene itself, among others, and is critical for *V. cholerae* virulence. We examined the effects of single and double point mutations on the ability of ToxT to activate *tcpA* transcription by constructing a collection of *tcpA*::*lacZ* fusions. Each fusion contained the region upstream of *tcpA* previously shown to be important for ToxT function (Hulbert and Taylor, 2002; Yu and DiRita, 2002). These plasmid-based fusions allowed us to test a large number of mutations; however, it is formally possible that there are differences in the requirements for ToxT to activate transcription of plasmid-based constructs and the requirements for activation on the *V. cholerae* chromosome.

The experimental results from *tcpA::lacZ* constructs having double point mutations gave us an indication of the general DNA segments that are important for ToxT-directed transcription of *tcpA*. Double mutations to two of the three T tracts in the *tcpA* promoter region, the promoter-proximal (tract 1) and promoter-distal (tract 3) T tracts, abrogated activation by ToxT. This finding is consistent with the presence of two ToxT binding sites, each of which contains one of the outer T tracts. The two binding sites are similar in sequence to those we have identified previously at other genes within the ToxT regulon (Fig. 6A) (Withey and DiRita, 2005a,b). The significant difference between the *tcpA* ToxT binding sites and the others we previously identified is that at *tcpA* the sites are configured as a direct repeat. Some other genes within the ToxT regulon also use toxboxes configured as a direct repeat. Our assignment of the binding sites at *ctxAB* (Fig. 6B) is based on data from the study by Yu and DiRita (2002); because of the T tract repeats in this sequence, we speculate that ToxT binds a direct repeat here as well. We also have evidence that ToxT activates two separate promoters at *tcpI* (Fig. 6A and B); one of these promoters, $P_{tcpI\text{-}1}$, has toxboxes configured as an inverted repeat and

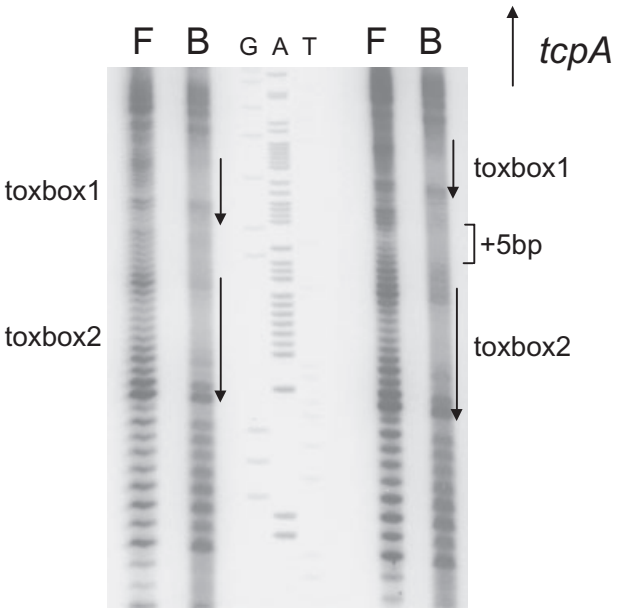


Fig. 5. Copper-phenanthroline footprinting of ToxT on DNA having a 5 bp insertion between the toxbox1 and toxbox2 sites. The results using wild-type *tcpA* DNA are shown at the left of the figure and the results using *tcpA* with a 5 bp insertion between the ToxT binding sites are shown at the right of the figure. Toxbox1 and toxbox2 are indicated by arrows. F = free DNA, B = ToxT/DNA complex. The brackets and '+5' indicate the position of the 5 bp insertion described in the text. The letters 'G, A, T' refer to the respective lanes of the sequencing ladder in the centre of the figure.

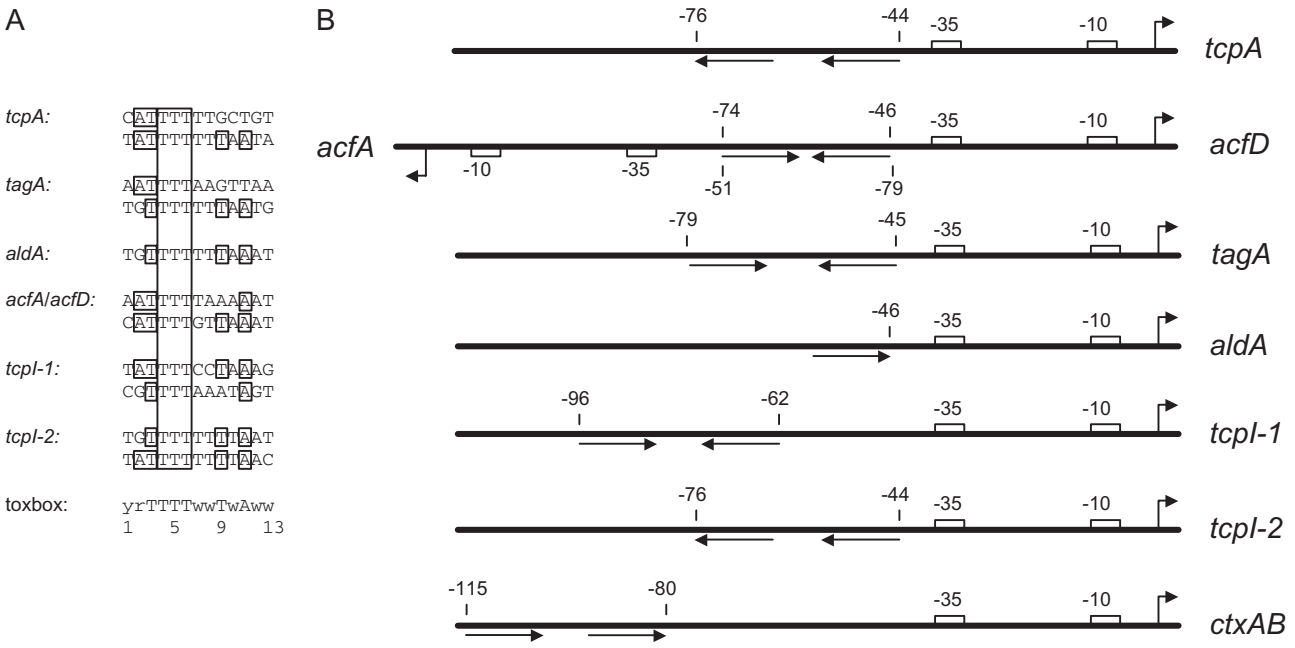


Fig. 6. Comparison of ToxT binding site sequences and arrangements among ToxT regulon genes.
A. Alignment of ToxT binding sites. The gene names are at the left of the figure, and the toxbox sequences located upstream of each gene are at the right. The solid boxes indicate the core toxbox determinants. The consensus toxbox sequence is shown at the bottom of the figure, as is the numbering of the toxbox positions described in the text. In the consensus toxbox sequence, 'r' indicates that the nucleotide can be either A or G, 'w' indicates that the nucleotide can be either A or T and 'y' indicates that the nucleotide can be either C or T.
B. Arrangements of toxboxes upstream of ToxT regulon genes. The core promoter –10 and –35 elements are indicated by boxes. Toxboxes are indicated by arrows. Numbers refer to the distances upstream of the start sites of transcription.

the other, $P_{tcpI\text{-}2}$, has toxboxes configured as a direct repeat (J.H. Withey and V.J. DiRita, in preparation).

Double mutations to the central T tract (tract 2) caused a reduction but not an abrogation of *tcpA* activation by ToxT. Because A tracts are known to cause narrowing of the DNA minor groove and a curvature or kink in the DNA structure (Wing *et al*., 1980; Burkhoff and Tullius, 1987; Haran and Crothers, 1989), it is plausible that the A tract corresponding to T tract 2 has a structural role in activation of *tcpA* by ToxT by permitting enhanced contact between the ToxT molecule bound to the promoter-distal binding site and RNAP. Another possibility would be that this central A tract increases interaction between ToxT monomers bound to the two sites, again by bending DNA. A recent report suggests that ToxT may dimerize or that monomers may interact at *tcpA* (Prouty *et al*., 2005). We present evidence here and elsewhere (Withey and DiRita, 2005a) that ToxT likely binds to DNA as a monomer even when multiple binding sites are required for its function as an activator. However, interactions between ToxT monomers after DNA binding may be required for activation of *tcpA* transcription; this would be one explanation for our observation that the 5 bp and 10 bp insertions between the two ToxT sites abrogate activation of *tcpA* transcription by ToxT (Fig. 5).

The experimental results from *tcpA::lacZ* constructs having single point mutations gave us the first clear indication of what specific sequences are required for activation of *tcpA* transcription by ToxT. We identified three consecutive T nucleotides within both toxbox1 and toxbox2 that are required for significant ToxT-directed transcription of *tcpA*. These three T nucleotides are at the same relative positions of both toxbox1 and toxbox2, i.e. nucleotides 4–6 (Figs 1 and 6A). Mutations to other positions within toxbox1 did not cause significant defects in activation by ToxT. However, mutations to positions 9 and 11 of toxbox2 caused dramatic defects in activation by ToxT, and mutations to positions 2 and 3 of toxbox2 caused significant defects in activation by ToxT. There are two notable observations based on this information: (i) the ToxT binding site is degenerate; at most the identities of seven of 13 bp are important for ToxT function and (ii) comparison of the toxbox1 and toxbox2 sites indicates a difference in ToxT requirements; the toxbox1 site has three nucleotides at which mutations cause significant defects in activation by ToxT, whereas the toxbox2 site has seven nucleotides at which mutations cause significant defects in activation by ToxT. The differences in sequence translate directly to differences in protection in footprinting experiments with *tcpA* promoter DNA; toxbox2 is pro-

tected along its entire length, including critical positions 2–6, 9 and 11, whereas toxbox1 is protected only in the region of the T tract (Fig. 4A and B). Similar differences in copper-phenanthroline footprinting were also observed for other toxboxes (Withey and DiRita, 2005a,b). This suggests ToxT is making specific contacts with the DNA at the seven critical base pairs of the toxbox, as defined by the mutations to toxbox2, and that altering the DNA sequence removes the specific interaction and thus the protection conferred by ToxT in copper-phenanthroline footprinting experiments. In support of this hypothesis, mutations to the *acfA* and *acfD* toxboxes that cause a loss in transcription activation by ToxT also cause a reduction in ToxT protection to base pairs in close proximity to the mutations, although DNA binding by ToxT is retained (Withey and DiRita, 2005a).

The results from constructs having single point mutations and constructs having double point mutations are consistent with regard to which base pairs are required for ToxT to activate transcription; mutations to any of the base pairs shown to be most important for ToxT-directed *tcpA* transcription in Figs 2 and 3 also caused severe defects in activation by ToxT when a second base pair was also mutated. Neither single nor double point mutations to positions 7–13 of toxbox1 caused defects in activation by ToxT. All of the single base pair mutations described in this report change A/T base pairs to G/C base pairs; this was done to maximize the effects of a single mutation in an effort to determine which base pairs are most critical for ToxT function. It is possible that changing an A/T base pair to T/A would have a lesser effect on activation by ToxT.

Based on this new information about sequence requirements for DNA binding and activation by ToxT, we propose that the ToxT binding site be termed the 'toxbox', in accordance with the nomenclature for other AraC family proteins such as MarA (marbox) and SoxS (soxbox) that bind to degenerate DNA sequences (Martin *et al*., 1999; Dangi *et al*., 2001; Griffith and Wolf, 2001; Martin and Rosner, 2001). Comparison of the toxboxes found at *tcpA* with those we have identified at other ToxT-activated genes (Withey and DiRita, 2005a,b) suggests that the sequence requirements are similar at all toxboxes, particularly at those positions that we found were critical for activation of *tcpA* by ToxT (Fig. 6A). We refer to the seven critical nucleotides at positions 2–6, 9 and 11 of the toxbox, as indicated by the toxbox2 mutations, as the core toxbox determinants.

The *Escherichia coli* MarA, SoxS, and Rob proteins are all about 50% identical in amino acid sequence, bind to DNA and activate transcription as monomers, and recognize the same degenerate 20 bp sequence (Martin *et al*., 1999; Griffith and Wolf, 2001). Because a large number of sequences consistent with the marbox consensus sequence are found within the *E. coli* chromosome, if specific recognition of the marbox sequence were the only means of determining MarA, SoxS, or Rob binding, these proteins would rarely find binding sites at the appropriate positions relative to a promoter, and would be swamped by potential binding sites on the chromosome. Instead it has been proposed that complexes are formed between the activator proteins and RNAP prior to DNA binding, and that the activator/RNAP complexes then search for activator binding sites adjacent to promoters; this is referred to as 'prerecruitment' (Griffith *et al*., 2002) or 'DNA scanning' (Martin *et al*., 2002). It is quite possible that ToxT uses a similar mechanism to identify its appropriate binding sites, as it would be similarly confronted with abundant sequences similar to actual toxboxes within the *V. cholerae* chromosome.

All of the known genes within the ToxT regulon have toxboxes located upstream of the –35 box, suggesting that each gene has a class I promoter (Fig. 6B) and thus ToxT most likely interacts with the RNAP α-CTD (Busby and Ebright, 1994). Our observations that ToxT activates transcription of various promoters using several different toxbox configurations suggests that there is significant flexibility in the putative interactions between ToxT and α-CTD. We propose here that the architecture of ToxT-activated promoters with regard to ToxT and α-CTD differs depending on the configuration of toxboxes relative to the promoter (Fig. 7). At promoters having two toxboxes, the promoter-proximal toxbox is always in the same orientation relative to the promoter (Fig. 6B). Thus, interactions between ToxT bound at this position and one α-CTD would be the same regardless of the orientation of the promoter-distal toxbox (Fig. 7). The position of the other α-CTD would differ depending on whether toxboxes are configured as a direct or inverted repeat. In the case of *aldA*, which has only one toxbox that is oriented differently than other promoter-proximal toxboxes, the interaction between ToxT and α-CTD would resemble that of the promoter-distal toxbox in an inverted repeat (Fig. 7).

Previous studies on ToxT activation of *acfA* and *acfD* addressed the question of whether ToxT acts as two monomers or a dimer to activate transcription of those genes (Withey and DiRita, 2005a). Numerous AraC/XylS family members bind DNA either as monomers (Martin *et al*., 1999; Griffith and Wolf, 2001; Ibarra *et al*., 2003) or as dimers (Egan and Schleif, 1994; Schleif, 2000). Footprinting experiments using mutant DNA with 5 bp or 10 bp insertions between the ToxT binding sites, which would rotate the binding sites approximately one half-turn or one full turn of the DNA helix relative to each other, respectively, showed that ToxT protects sites with altered spacing in a manner indistinguishable from the protection conferred to sites having wild-type spacing. This strongly suggests that the individual toxboxes between *acfA* and *acfD*

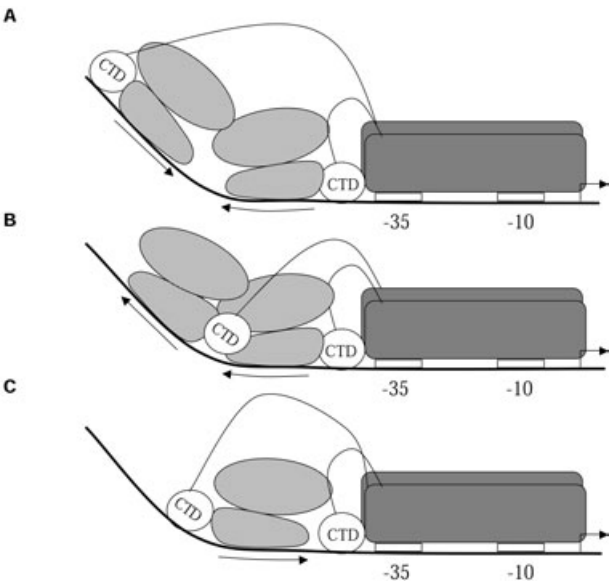


Fig. 7. Models for promoter architecture at genes having different toxbox configurations. The stippled ovals indicate ToxT, the white circles indicate the RNAP α-CTD, the dark ovals indicate the rest of RNAP, the −10 and −35 promoter elements are indicated by boxes and labelled, and arrows indicate toxbox positions and orientations.
A. Architecture at a promoter having two toxboxes in an inverted repeat configuration (*acfA*, *acfD*, *tagA*, *tcpI-1*).
B. Architecture at a promoter having two toxboxes in a direct repeat configuration (*tcpA*, *tcpI-2*, *ctxAB*).
C. Architecture at a promoter having one toxbox (*aldA*).

do not represent half sites of a larger ToxT binding site, as each toxbox can be bound independently, presumably by a ToxT monomer. The arrangement of toxboxes in the *tcpA* promoter region is different (direct repeat) than in the *acfA-acfD* intergenic region (inverted repeat), so it is possible that ToxT dimerization is required for DNA binding and transcription activation of *tcpA*. Our results here from a similar copper-phenanthroline footprinting experiment in which 5 bp was inserted between the *tcpA* toxboxes indicated that both of the *tcpA* toxboxes were protected by ToxT regardless of their position relative to each other, again suggesting that single toxboxes may be bound by ToxT monomers. We did, however, observe some protection in the region between toxbox1 and toxbox2 having the 5 bp insert; the reasons for this are unclear.

The 5 bp and 10 bp insertions between toxbox1 and toxbox2 both abrogated activation of *tcpA* by ToxT. Because the 5 bp insertion would rotate toxbox2 one half-turn of the DNA away from the core promoter, it is likely that the loss of activation with this construct is due to the inability of ToxT bound to toxbox2 to interact productively with RNAP (Fig. 7). The 10 bp insertion would rotate the toxboxes approximately one full turn of the DNA relative to each other and could restore the ability of the α-CTD to contact the distal ToxT; the observation that this insertion abrogates transcription suggests that the spacing of the binding sites relative to each other, relative to the promoter, or both is important, or that this insertion alters the local DNA structure. The 10 bp insertion does disrupt the central T tract, and thus likely prevents the DNA bend that may normally be found there. We have further observed that the 5 bp and 10 bp insertions between the −35 promoter element and the promoter-proximal toxbox at *acfA* also abrogate activation by ToxT (J.H. Withey and V.J. DiRita, unpubl. data), further suggesting that proper spacing of the toxboxes relative to the promoter is important for activation by ToxT. Another possibility is that ToxT monomers interact with each other subsequent to DNA binding and that this interaction is necessary for transcription activation of *tcpA* by ToxT; this idea is consistent with recent work that showed dimerization activity in the ToxT NTD (Prouty *et al*., 2005). A final possibility is that ToxT dimers are binding at each toxbox but that no protection is conferred by half of each ToxT dimer. However, we think it unlikely that a single ToxT dimer binds to a single pair of toxboxes given the variety in toxbox configuration and orientation observed at different promoters. Future work will address the position, orientations and interactions of ToxT and RNAP α-CTD at various promoters and test the models that we have proposed in Fig. 7.

In summary, we have identified specific sequence requirements for ToxT to activate transcription of the *V. cholerae tcpA* operon. The ToxT DNA binding sites, or toxboxes, have a degenerate sequence within which the identity of only a few base pairs, the core toxbox determinants, is important. Accordingly, there is considerable conservation of sequence of these core determinants among all known ToxT binding sites. ToxT uses two toxboxes in a direct repeat configuration to activate transcription of *tcpA* and likely binds to each toxbox as a monomer. This configuration differs from the ToxT binding site configurations that we have described previously at other genes; in these cases inverted repeat or single binding sites are found. ToxT thus is extremely flexible in terms of both the sequences to which it binds and the configurations of binding sites from which it activates transcription.

Experimental procedures

Bacterial strains and plasmids

All strains used in this study are derivatives of O395 (wild-type *toxT*, DiRita lab collection) or VJ740 (Δ*toxT*, Champion *et al*., 1997) carrying *tcpA::lacZ* fusions on plasmid pTL61T (Linn and St Pierre, 1990). Strains were grown at 37°C in Luria broth (LB) medium for overnight cultures, and at 30°C in LB adjusted to a starting pH of 6.5 (inducing conditions) for use in β-galactosidase assays. Strains were maintained at −70°C in LB + 20% glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; streptomy-

cin, 100 μg ml^{-1}. Plasmids were introduced into *V. cholerae* strains by electroporation using a Bio-Rad *E. coli* Pulser.

DNA manipulations

Plasmids were purified using the Qiagen Spin Miniprep or Plasmid Midi kits. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase from Roche as specified by the manufacturer. Restriction enzymes were purchased from New England Biolabs and used as specified by the manufacturer.

Plasmid construction

The wild-type *tcpA::lacZ* fusion was constructed by PCR of the appropriate region using fresh O395 colonies as template. Site-directed mutations were created using the splicing by overlap extension technique (SOE) (Horton *et al*., 1989; Horton *et al*., 1993), after which inserts having the desired mutations were cloned between the XbaI and HindIII sites of pTL61T (Linn and St Pierre, 1990). The nucleotide sequences of all plasmid constructs were confirmed by DNA sequencing at the University of Michigan Sequencing Core.

β-Galactosidase assays

For β-galactosidase assays, *V. cholerae* strains were grown overnight at 37°C, subcultured at a 1:40 dilution into fresh LB, pH 6.5, and grown for 3 h at 30°C. Bacteria were then placed on ice and chloramphenicol was added to 0.5 mg ml^{-1}. Assays were performed according to the method of Miller (Miller, 1972).

Copper-phenanthroline footprinting

Electrophoretic mobility shift assay (EMSA) was performed as previously described (Withey and DiRita, 2005a). The amount of H_6-ToxT used was determined empirically to be the amount required to shift approximately 50% of the labelled DNA. Identical amounts of H_6-ToxT were added to both wild-type DNA probes and DNA probes having +5 bp insertions as detailed in the text, and both produced similar amounts of bound product. Plasmids used in the β-galactosidase assays were used as PCR templates as indicated in the text. After EMSA, the procedure used was that of Papavassiliou (Papavassiliou, 1994). Briefly, the gel was soaked in 200 ml 10 mM Tris-HCl, pH 8 in a glass tray. One millilitre of 40 mM 1,10-Phenanthroline (Sigma) was mixed with 1 ml 9 mM $CuSO_4$ (Sigma) for 1 ml, then diluted with 18 ml dH_2O. This was added to the gel tray and mixed by shaking. A 20 ml aliquot of a 1:200 dilution of 3-Mercaptopropionic acid (Sigma) was then added to the gel tray and briefly mixed. The reaction continued for 7 min, and was stopped by addition of 20 ml 28 mM Neocuproine (Sigma), followed by shaking for 2 min. The gel was rinsed with 1000 ml dH_2O and placed on X-ray film for 3 h. After the film was developed, bands corresponding to free DNA and H_6-ToxT/DNA complexes were excised from the gel based on their location in the film, and the gel slices were crushed and the DNA eluted overnight in 0.5 ml 0.5 M Ammonium Acetate, pH 7.5, 1 mM EDTA, 0.1% SDS, 10 mM $MgCl_2$. Gel pieces were pelleted by centrifugation, and the supernatant was passed through a 0.2 μm syringe filter and ethanol precipitated. The pellets were resuspended in a 1:1 mixture of TE : sequencing stop solution (USB) and radioactivity was measured with a Geiger counter. Approximately equal amounts of labelled DNA from the free DNA and H_6-ToxT/DNA complex bands were loaded on the subsequent sequencing gel. The sequencing ladder was produced with a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB) as specified by the manufacturer, and the sequencing gel was prepared and run as specified by the sequencing kit manual. The same plasmid template and the primer that was end-labelled in the EMSA/footprinting experiment were used in the sequencing reactions. Autoradiography was performed with the resulting gel, and typical exposure times were 10–14 days.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Double point mutations to the *tcpA* ToxT binding region. The mutated nucleotides are shown in bold. Only the template strand sequence (lower strand in Fig. 1) is shown. The results of β-galactosidase assays in wild-type and Δ*toxT* strains carrying plasmids with the indicated mutations upstream of the *tcpA::lacZ* fusion are shown to the right of the sequences. 'Activation' indicates the fold-difference in β-galactosidase activity between the wild-type *toxT* and Δ*toxT* strains for that construct. A minimum of three separate experiments were performed for each strain, and the values shown are the mean ± the standard deviation.

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