Activation of both acfA and acfD transcription by *Vibrio cholerae* ToxT requires binding to two centrally located DNA sites in an inverted repeat conformation

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Summary

The Gram-negative bacterium *Vibrio cholerae* is the infectious agent responsible for the disease Asiatic cholera. The genes required for *V. cholerae* virulence, such as those encoding the cholera toxin (CT) and toxin-coregulated pilus (TCP), are controlled by a cascade of transcriptional activators. Ultimately, the direct transcriptional activator of the majority of *V. cholerae* virulence genes is the AraC/XylS family member ToxT protein, the expression of which is activated by the ToxR and TcpP proteins. Previous studies have identified the DNA sites to which ToxT binds upstream of the ctx operon, encoding CT, and the tcpA operon, encoding, among other products, the major subunit of the TCP. These known ToxT binding sites are seemingly dissimilar in sequence other than being A/T rich. Further results suggested that ctx and tcpA each has a pair of ToxT binding sites arranged in a direct repeat orientation upstream of the core promoter elements. In this work, using both transcriptional lacZ fusions and *in vitro* copper-phenanthroline footprinting experiments, we have identified the ToxT binding sites between the divergently transcribed acfA and acfD genes, which encode components of the accessory colonization factor required for efficient intestinal colonization by *V. cholerae*. Our results indicate that ToxT binds to a pair of DNA sites between acfA and acfD in an inverted repeat orientation. Moreover, a mutational analysis of the ToxT binding sites indicates that both binding sites are required by ToxT for transcriptional activation of both acfA and acfD. Using copper-phenanthroline footprinting to assess the occupancy of ToxT on DNA having mutations in one of these binding sites, we found that protection by ToxT of the unaltered binding site was not affected, whereas protection by ToxT of the mutant binding site was significantly reduced in the region of the mutations. The results of further footprinting experiments using DNA templates having +5 bp and +10 bp insertions between the two ToxT binding sites indicate that both binding sites are occupied by ToxT regardless of their positions relative to each other. Based on these results, we propose that ToxT binds independently to two DNA sites between acfA and acfD to activate transcription of both genes.

Introduction

The severe diarrhoal disease cholera is caused by the Gram-negative, comma-shaped bacterium *Vibrio cholerae*. *V. cholerae* is usually acquired by ingestion of contaminated food or water (Finkelstein, 1973). The primary factor required for cholera disease is the cholera toxin (CT), an AB toxin encoded by the ctxAB genes (Lonnroth and Holmgren, 1973; Gill, 1976). Cholera toxin enters the intestinal epithelium by binding, with its five B subunits, to ganglioside GM1, followed by transport of the one active A subunit into the host cell (Holmgren et al., 1973). The A subunit activates adenylyl cyclase, resulting in a large increase in cyclic AMP (cAMP) levels. This induces secretion of fluid into the lumen of the intestine to produce the voluminous watery diarrhoea ('rice water stool') that is characteristic of cholera disease (Field et al., 1972). ctxAB are carried within the genome of a lysogenic filamentous bacteriophage, CTXΦ (Waldor and Mekalanos, 1996). Colonization of the intestine by *V. cholerae* requires the toxin-coregulated pilus (TCP), a type IV pilus encoded by genes within the tcpA operon, which is expressed under the same environmental conditions as CT (Taylor et al., 1987; Peterson and Mekalanos, 1988). TCP is also the receptor for CTXΦ (Waldor and Mekalanos, 1996). The genes encoding the TCP are located on the large *V. cholerae* chromosome within the vibrio pathogenicity island (VPI) (Peterson and Mekalanos, 1988; Kaufman et al., 1993; Ogierman et al., 1993; Karaolis et al., 1998).

Expression of CT and TCP is subject to control by a complex network of positive and negative transcriptional
regulators. The direct activator of transcription of the genes encoding both CT and TCP is ToxT, a 32 kDa cytoplasmic protein (DiRita et al., 1991; Higgins et al., 1992). Expression of toxT from its own promoter is initiated by the combined activity of two membrane-localized transcriptional activators, ToxR and TcpP (Hase and Mekalanos, 1998; Krukonis et al., 2000; Krukonis and DiRita, 2003). These proteins, along with their protein cofactors, ToxS and TcpH, respectively, bind to DNA upstream of toxT and activate toxT transcription. Further expression of toxT is then induced by ToxT itself through positive feedback, via its role in activating transcription of the tcpA operon, within which the toxT gene is located (Higgins and DiRita, 1994; Brown and Taylor, 1995; Yu and DiRita, 1999). Expression of the tcpPH operon, which is also located within the VPI, is positively regulated by the transcriptional activators AphA and AphB, and negatively regulated by the cAMP receptor protein (CRP) and PepA protein (Kovacikova and Skorupski, 1999; 2001; 2002a; Skorupski and Taylor, 1999; Behari et al., 2001). Also implicated in this cascade is HapR, whose activity is controlled by the quorum-sensing system, and which negatively regulates expression of aphA (Jobling and Holmes, 1997; Kovacikova and Skorupski, 2002b; Miller et al., 2002; Zhu et al., 2002; Kovacikova et al., 2003).

ToxT protein is a member of the AraC/XylS family of transcriptional regulators (Higgins et al., 1992). AraC/XylS family members have in common a conserved DNA binding and transcriptional activation domain of approximately 100 amino acids, which contains two helix–turn–helix motifs (Gallegos et al., 1997; Martin and Rosner, 2001; Tobes and Ramos, 2002). Crystal structures that include the conserved domain have been determined for two AraC/XylS family members, MarA and Rob (Rhee et al., 1998; Kwon et al., 2000). The crystal structures of MarA and Rob bound to DNA suggest that the modes of DNA binding utilized by these two proteins differ; the structure of MarA bound to DNA indicates that both helix–turn–helix motifs specifically interact with the major groove, whereas the structure of Rob bound to DNA indicates that only the more N-terminal helix–turn–helix interacts specifically with the major groove. ToxT carries the 100-amino-acid AraC/XylS domain as its C-terminal domain. However, ToxT also has a 176-amino-acid N-terminal domain of unknown function. Other members of the AraC/XylS family having a second domain typically use this domain for multimerization and/or effector binding (Gallegos et al., 1997; Martin and Rosner, 2001; Tobes and Ramos, 2002). Whether ToxT is monomeric, as are some AraC/XylS family members, or forms dimers or multimers in solution, as do other AraC/XylS family members, has not been determined. The stoichiometry of ToxT binding to DNA has also not been determined.

Previous studies on ToxT function have focused primarily on the role of ToxT in activating expression of ctx and tcpA. ToxT was found to have dual roles in activating expression of ctx (Yu and DiRita, 2002). First, ToxT acts as an anti-repressor of ctx. It does so by competing for binding at DNA sites upstream of ctx with H-NS protein, which has multiple binding sites located in this region and acts to reduce expression of ctx (Nye et al., 2000). Second, ToxT acts as a direct activator of ctx transcription, presumably by interacting with RNA polymerase. In contrast to its negative effect upon ctx expression, H-NS has a minimal effect upon tcpA expression (Nye et al., 2000; Yu and DiRita, 2002). Therefore, at tcpA, ToxT acts not as an anti-repressor but only as a direct activator of transcription. This is most probably through interaction between ToxT and the C-terminal domain(s) of the α-subunits of RNA polymerase (α-CTD) (Hulbert and Taylor, 2002; Yu and DiRita, 2002).

The ToxT binding sites upstream of the ctx and tcpA core promoter elements were identified using both genetic methods and DNase I footprinting experiments (Hulbert and Taylor, 2002; Yu and DiRita, 2002). The sequences of these ToxT binding sites are somewhat dissimilar from each other, and a consensus sequence is not evident. The notable common features found in both the ctx and tcpA ToxT binding sites are poly A or poly T tracts of five or more contiguous A or T nucleotides on one strand of the DNA. The ToxT binding sites upstream of ctx have seven of these tracts; the ToxT binding sites upstream of tcpA have three of these tracts. Mutational analyses of the ToxT binding sites located upstream of tcpA using either a genetic screen (Hulbert and Taylor, 2002) or a site-directed approach (J.H. Withey and V.J. DiRita, in preparation) indicated that the promoter-proximal and promoter-distal A/T tracts are required for ToxT-dependent transcription of tcpA. Mutations in the central A/T tract had little to no effect on ToxT-directed transcription of tcpA. These results are consistent with ToxT binding to two sites upstream of tcpA in a direct repeat orientation, with each site containing one A/T tract.

In addition to its role in activating expression of the ctx and tcpA operons, ToxT also activates expression of at least five other genes: acfA, acfD, aldA, tagA and tcpI (Peterson and Mekalanos, 1988; Parsot and Mekalanos, 1991; Parsot et al., 1991). All of these genes are located within the VPI. As was found for ctx and tcpA, poly A or poly T tracts are found upstream of each of these genes, and these are the only obvious similarities in the DNA sequences upstream of these genes, which presumably contain ToxT binding sites. acfA and acfD are divergently transcribed genes that encode components of the accessory colonization factor (ACF) (Peterson and Mekalanos, 1998). The exact role of the ACF in pathogenesis is unclear, but it is required by V. cholerae for efficient colo-

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nization of the infant mouse small intestine. aldA and tagA are also divergently transcribed genes. aldA encodes an aldehyde dehydrogenase (Parsot and Mekalanos, 1991). tagA encodes a putative transmembrane protein (Harkey et al., 1995). The roles, if any, of aldA and tagA in pathogenesis are unknown. tcpI encodes a putative methylaccepting chemotaxis protein (Harkey et al., 1994). Again, the role, if any, of tcpI in pathogenesis is unknown.

In the studies described in this report, we used a combination of mutational analysis and copper-phenanthroline footprinting experiments to identify sites located between acfA and acfD that are required for ToxT binding and transcriptional activation. These ToxT binding sites are arranged in an inverted repeat orientation; this is in contrast to the previously described ToxT binding sites at tcpA and ctx, which are in direct repeat orientations. However, in similarity to ctx and tcpA, each of the acfA/acfD ToxT binding sites contains one A/T tract. Occupancy of both ToxT binding sites is required for transcription of both acfA and acfD. Mutation of one of the two binding sites does not significantly affect occupancy by ToxT of the unaltered binding site. Furthermore, both sites remain occupied by ToxT even when the spacing between the two binding sites is altered by the insertion of 5 or 10 base pairs (bp). Based on these results and previous work on other members of the AraC/XylS family, we propose that one ToxT monomer binds independently to each of the two binding sites between acfA and acfD, and that both ToxT monomers are required to make contacts with the RNA polymerase α-CTDs to activate transcription of acfA and acfD.

Results

Identification of the minimal acfA promoter region

There is a near consensus −10 sequence (TATAAT) found upstream of both genes, the spacing of which is in good agreement with where primer extension experiments suggested each transcript initiates (data not shown). However, both genes have a degenerate putative −35 sequence at the appropriate position relative to the −10 element (Fig. 1). To determine the length of DNA upstream of the core promoter elements of acfA that is required for ToxT-directed transcription, we constructed nested acfA::lacZ fusions in which the 5′ end point of DNA upstream from the transcription start site for acfA varies from −146 to −66 (Fig. 2). These fusions were constructed in plasmid pTL61T, which has a promoterless lacZ gene downstream of a multiple cloning site (Linn and St Pierre, 1990). The effect of ToxT on expression of the fusions was determined by measuring β-galactosidase activity in wild-type and toxT mutant V. cholerae strains (O395 and VJ740 respectively; Champion et al., 1997).

ToxT-dependent acfA–lacZ expression was observed with fragments harbouring DNA upstream of and including residue −88 (Figs 2 and 3). The next deletion in the series, acfA4, with its end point at −66, was inactive irrespective of the presence of ToxT. Deletion to −88 (acfA3) apparently removes a site that inhibits ToxT from activating the acfA promoter, as the acfA3 construct expressed about two and a half times more ToxT-dependent β-galactosidase activity than either of the other two fusions with end points upstream of −88 (acfA1 and acfA2). This was not accompanied by an elevated basal level of transcription (i.e. in the absence of ToxT) and thus the behaviour of this promoter is distinct from that previously observed with the ctx promoter (Yu and DiRita, 2002). In that analysis, deletion of specific upstream sequences led to increased basal transcription and a concomitantly increased level of ToxT-activated transcription (Yu and DiRita, 2002). This was attributed to the effects of the nucleoid-like protein HNS, which is known to antagonize ToxT-dependent activation of the ctx promoter (Nye et al., 2000; Yu and DiRita, 2002).
These results indicate that DNA sequence information required for ToxT-directed transcription of acfA resides within 88 nucleotides upstream of transcription initiation. The promoter is also subject to some measure of inhibition through sequence information between −115 and −88 (Fig. 3).

**Mutational analysis of the acfA promoter region**

The most significant similarities in sequence upstream of all the known ToxT-controlled genes are the presence of tracts of A or T nucleotides, so these tracts upstream of acfA were targeted for mutagenesis to assess their role in ToxT-directed transcription of acfA. Site-directed mutations were created in derivatives of the minimal ToxT-directed acfA::lacZ fusion, acfA3, which ends at position −88 (Fig. 1). The most dramatic effects were produced by changing the base pairs at −74/−75 and −54/−56 from A/T to G/C (acfA13) and by changing the base pairs at −69 and −67 to G/C (acfA16) (Fig. 3). In each case, the fusion was expressed to the same, low level in both the wild-type and ΔtoxT backgrounds, suggesting that the base pairs at −74/−75 and −54/−56 are required for ToxT-directed transcription of acfA.

Two other mutations we introduced affected the basal (ToxT-independent) expression of the fusion alleles slightly, but we can nevertheless draw some conclusions about the requirements for the altered residues in ToxT-dependent activation. Allele acfA17 exhibited a slightly higher baseline level of activation that was stimulated when ToxT was present (Fig. 3). The absolute level of ToxT-dependent expression was lower than the wild-type acfA4 allele, and thus we conclude that the residues at −69 and −67 are important, but not essential, for ToxT to activate. The elevated activity in the absence of ToxT was not explored further. In acfA15, fusion activity in the toxT mutant background was somewhat lower than we saw with acfA3 but, again, this allele was significantly stimulated in the presence of ToxT (Fig. 3). Activation of acfA15 was the lowest of any of the mutated alleles that still
exhibited ToxT-directed transcription, and thus, as with acfA17 we conclude that the A/T residues mutated in this allele (at −59 and −61) are important for, but not absolutely critical to, ToxT-dependent expression.

Finally, changing the base pairs at −82 and −83 from A/T to G/C, to create acfA14, had a minimal effect, if any, on transcription. As expected, mutations to A or T tracts located upstream of −88 had no effect on ToxT-directed transcription of acfA (data not shown).

**Identification of the minimal acfD promoter region**

We used a method similar to that described above for acfA to determine the length of DNA upstream of the core promoter elements of acfD that is required for ToxT-directed transcription. Nested lacZ fusions were constructed in pTL61T, and β-galactosidase levels were assayed in strains carrying these fusions and having either wild-type toxT or a deletion in toxT. This series extended from −139 bp upstream to −66 bp upstream of the transcription start site (Fig. 2). The first observation to note is that the ratio of ToxT-dependent to basal expression of acfD is much greater than that of acfA, reflecting primarily a lower basal promoter activity for acfD (Fig. 4).

The wild-type toxT strain carrying acfD1 produced 24-fold more β-galactosidase activity than the ΔtoxT strain carrying acfD1 (Fig. 4). Deletion of the DNA sequence between −139 and −87 (acfD2) had no effect on ToxT-directed transcription of acfD. However, truncation of the DNA sequence to −66 (acfD3) abrogated ToxT-directed transcription of acfD (Fig. 4). Therefore, the sequence between −87 and −66 relative to the start of acfD transcription must contain determinants for ToxT-directed transcription of acfD (Fig. 1), and the region from −87 to +12 is sufficient for ToxT-directed transcription of acfD. The minimal acfA4::lacZ and acfD2::lacZ fusion constructs that exhibit ToxT-directed transcription of these genes overlap by 52 bp (Figs 1 and 2), suggesting that a single ToxT control region may exist between acfA and acfD.

**Mutational analysis of the acfD promoter region**

The A and T-rich sequences upstream of acfD were also targeted for mutation to determine their importance to ToxT-directed transcription of acfD. Site-directed mutations were created in derivatives of the minimal ToxT-directed acfD::lacZ fusion, acfD2, which ends at position −87 (Fig. 1) and is activated over 30-fold by ToxT. Mutations having the strongest negative effects on ToxT-directed transcription of acfD2 were A/T to G/C alterations at −70/−68 (acfD11), −50/−49 (acfD13) and −74/−72 (acfD14). All of these mutant alleles expressed ToxT-dependent β-galactosidase at levels barely above background, and had toxT/ΔtoxT ratios over 15 times lower than the wild-type allele. Thus, it is clear that these residues are essential for ToxT-dependent activation of acfD.

The other two mutant alleles we investigated in this series could still be activated by ToxT, but the level of activation was much lower than the acfD2 allele. These are acfD12, with A/T to G/C changes at −65 and −63, and acfD15, with these changes at −57 and −55. acfD15 in particular was still activated over eightfold by ToxT, but this level of activation is significantly lower than the 33-fold activation seen with acfD2. We conclude that these four residues are required for maximum ToxT-dependent expression, but that even without them ToxT remains capable of some degree of activation.

The mutations with the largest negative effect on ToxT-directed acfD transcription, acfD11 and acfD13, are identical mutations to those in alleles acfA16 and acfA13.

![Fig. 4. Results of β-galactosidase assays on strains carrying acfD::lacZ fusions. The results of experiments performed with V. cholerae strains having wild-type ToxT (O395) are represented as grey bars, and the results of experiments performed with V. cholerae strains having a toxT deletion (VJ740) are represented as white bars. 'Allele' indicates which acfD::lacZ fusion construct was used in the experiment; '5' end point' indicates the length of DNA upstream of the transcriptional start site that is present in the construct; and 'toxT/ΔtoxT' indicates the fold difference between the mean β-galactosidase values for that construct measured in O395 and measured in VJ740. β-Galactosidase assays were performed in each strain a minimum of three times, and the values shown are the mean ± standard deviation.](image_url)
respectively (Fig. 1), which were the most severely affected for ToxT-directed transcription (Figs 3 and 4). This suggests that ToxT activates transcription of both genes from the same central control region.

Identification of the ToxT binding sites between acfA and acfD by copper-phenanthroline footprinting

The genetic experiments described above allowed us to determine regions between acfA and acfD that are required for ToxT-directed transcription. Electrophoretic mobility shift analysis (EMSA) confirmed that purified ToxT binds specifically to the acfA–acfD intergenic DNA (data not shown) but did not provide any information regarding the stoichiometry of binding. This is because, as we have previously shown, ToxT–DNA complexes enter the gel poorly, and we are not able to resolve distinct complexes of bound monomers versus bound dimers (e.g. Yu and DiRita, 2002).

To more precisely identify the DNA sequence bound by ToxT we used DNA footprinting. Standard DNase I footprinting gave unclear results (data not shown), presumably because the DNA sequence between acfA and acfD has abundant A and T nucleotides, including numerous poly A tracts, and these are poorly cleaved by DNase I due to narrowing of the minor groove (Nelson et al., 1987; Urbach and Dervan, 2001). Instead, we used the copper-phenanthroline footprinting technique, in which chemical digestion of the DNA was performed after electrophoretic separation of the H₆-ToxT/DNA complexes and free DNA. The chemically digested protein/DNA complexes and free DNA were then eluted from the EMSA gel and run side by side on a sequencing gel beside a sequencing ladder to assess where H₆-ToxT had prevented chemical cleavage of the DNA, indicating a region of specific binding. As shown in Fig. 5, we observed a single region of protection between acfA and acfD conferred by H₆-ToxT, bracketed by hypersensitive sites at positions –49 and –77 relative to the acfA transcription start site or –47 and –75 relative to the acfD transcription start site. This protection is in the same DNA region we identified as being required for ToxT-directed transcription of both acfA and acfD in the lacZ fusion experiments described above. More specifically, the 5’ end points of the acfA4::lacZ and acfD3::lacZ fusion constructs, which do not exhibit ToxT-directed transcription, are within the DNA sequence protected by ToxT in these experiments, indicating that the failure of ToxT to activate transcription of these constructs results from disruption of the ToxT/DNA binding region. The strongest protection was seen in the area around the two A/T tracts, mutation of which completely abrogated activation of transcription by ToxT. The areas between the A/T tracts showed less complete protection (Fig. 5). The DNA sequence between acfA and acfD that is protected from chemical cleavage by ToxT contains a degenerate inverted repeat sequence, with 9 out of 13 nucleotides in each side of the repeat having the same identity (asterisks in Fig. 6A). The 5′ portion of each side of the repeat sequence contains a T tract, and the 3′ portion is A/T rich. Mutations to the 5′ T tract of either side of the repeat sequence abrogated ToxT-dependent transcription of both genes (acfA13, acfA16, acfD11, acfD13). Comparison of these ToxT binding sites to the previously

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identified ToxT binding regions upstream of \textit{ctx} and \textit{tcpA} shows conservation of the T tract in the 5’ half of the binding site (Fig. 6B). The 3’ portion of the binding sites are A/T rich, but have no specific sequence conservation. The most significant difference between the \textit{acfA} and \textit{acfD} ToxT binding sites and the \textit{ctx} and \textit{tcpA} ToxT binding sites is that the former are arranged in an inverted repeat orientation, whereas the latter are arranged in a direct repeat orientation (J.H. Withey and V.J. DiRita, in preparation).

**Mutations that prevent ToxT-directed transcription reduce specific binding of ToxT only to the mutated DNA binding site**

The results described above suggest that ToxT binds to two DNA sites in an inverted repeat configuration between \textit{acfA} and \textit{acfD}, and that occupancy of both of these DNA sites is required for ToxT-directed transcription of both \textit{acfA} and \textit{acfD}. We next asked how the occupancy of the putative ToxT binding sites is changed when one of the sites has mutations that prevent ToxT from activating transcription. EMSA experiments indicated that ToxT still binds specifically to the mutated DNA sites (data not shown). To further address this question, we performed copper-phenanthroline footprinting experiments on complexes of H$_2$-ToxT and DNA from mutants that are defective for ToxT-directed transcription of \textit{acfA} and/or \textit{acfD}. These mutants have alterations in either one or the other of the two putative ToxT DNA sites located between \textit{acfA} and \textit{acfD}. DNA from the \textit{acfA16} construct, which has mutations in the T tract of the \textit{acfA}-proximal ToxT binding site (and which has mutations identical to those in the \textit{acfD11} construct; Fig. 1), shows reduced protection by ToxT from chemical cleavage in the mutated binding site surrounding the location of the mutations (arrows in Fig. 7). However, the other ToxT binding site shows no apparent reduction in protection by ToxT. Likewise, DNA from the \textit{acfA17} construct, which has mutations in the 3’ A/T-rich portion of the \textit{acfA}-distal ToxT binding site (and which has mutations identical to those in the \textit{acfD15} construct; Fig. 1), shows reduced protection from chemical cleavage by ToxT in the mutant binding site (Fig. 7). The unaltered ToxT binding site, however, shows no apparent reduction in protection by ToxT. This mutant is particularly notable because it reduces ToxT-directed transcription of \textit{acfA} (\textit{acfA17}) but has little effect on ToxT-directed transcription of \textit{acfD} (\textit{acfD15}). These results strongly suggest that ToxT has two DNA binding sites between \textit{acfA} and \textit{acfD}.

**Insertions between the two ToxT binding sites do not abrogate ToxT binding to either site**

The above results indicating that mutations to one ToxT binding site do not affect protection by ToxT of the unaltered binding site raise the question of whether ToxT binds independently to the two sites between \textit{acfA} and \textit{acfD}. This question is made more compelling by our observations that ToxT uses a variety of binding site configurations to activate transcription of different genes. For \textit{acfA} and \textit{acfD}, as described above, and for \textit{tagA} (J.H. Withey and V.J. DiRita, in preparation), ToxT binds to two sites in inverted repeat configurations. For \textit{ctx} and \textit{tcpA}, ToxT binds to two sites in direct repeat configurations (J.H. Withey and V.J. DiRita, in preparation). And for \textit{aldA}, ToxT binds to a single site (J.H. Withey and V.J. DiRita, in preparation).

To address the question of whether ToxT binds independently to its two sites between \textit{acfA} and \textit{acfD}, we created derivatives of \textit{acfD2} having insertions of 5 bp or 10 bp between the two ToxT binding sites, creating the \textit{acfD21} and \textit{acfD22} constructs, respectively (Fig. 1), and performed copper-phenanthroline footprinting with H$_2$-ToxT on DNA amplified from these templates. We would predict that footprinting to one or both of the ToxT sites would be lost in the presence of a 5 bp insertion between the sites if interactions between the ToxT molecules bound to the two sites are required for binding. Because these constructs are derivatives of \textit{acfD2}, they show footprints on the opposite DNA strand from those presented earlier in this report using derivatives of \textit{acfA3} (Figs 5 and 7).

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**Fig. 6.** A. Sequence of the inverted repeat between \textit{acfA} and \textit{acfD}. The nucleotides that are identical in the two binding sites are indicated by asterisks above or below the sequence. Arrows represent the length and orientation of the putative binding sites. B. Comparison of the \textit{acfA}/\textit{acfD} ToxT binding sites to the ToxT binding sites previously identified upstream of \textit{tcpA} and \textit{ctx}. “Spacing” indicates the distance between the two ToxT binding sites. “Promoter proximity” indicates the promoter-proximal end of the nearest ToxT binding site relative to the start site of transcription. The consensus sequence is designated based on at least five out of the six binding sites having the indicated nucleotide at the indicated position. **W** signifies that the nucleotide can be either A or T.

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<th>Sequence: AATTTTTAAAAATTTAATAAATTTAACAAAATG</th>
<th>Repeat: Inverted</th>
<th>Spacing: 2bp</th>
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**Consensus:** AATTTTTxWWWxW

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Clear protection was conferred by H₆-ToxT in the area of the binding sites previously identified using acfA3 derivatives (Fig. 8, left). However, the hypersensitive sites produced by H₆-ToxT in footprints on acfA3 and derivatives (Figs 5 and 7) were not observed in footprints on acfD2 and derivatives. The 3′ end of the acfD-proximal binding site was also protected to a larger degree by H₆-ToxT in acfD2 and derivatives than in acfA3 and derivatives. The regions of DNA exhibiting H₆-ToxT-mediated protection were otherwise identical in footprints of both strands.

Clear protection was also conferred by H₆-ToxT to acfD21 DNA, which has 5 bp inserted between the two binding sites (Fig. 8, middle). Significantly, both binding sites were protected by H₆-ToxT, despite the fact that these sites have been rotated approximately one half turn of the DNA helix relative to their wild-type spacing. This strongly suggests that the ToxT molecules bound to the two binding sites are not interacting with each other. Finally, protection was also conferred by H₆-ToxT to acfD22 DNA, which has 10 bp inserted between the two binding sites (Fig. 8, right). To assess the effects of insertions between the binding sites on transcriptional activation by ToxT, β-galactosidase assays were performed on acfD21::lacZ and acfD22::lacZ fusions. Neither of these constructs was able to support ToxT-directed transcription (Fig. 9). This finding suggests that, although both of the ToxT binding sites are occupied in both the acfA21 and acfA22 constructs, this occupancy is not sufficient to allow ToxT to activate transcription. Therefore, the spacing of the binding sites relative to the promoter is an important factor in ToxT-directed transcription.

**Discussion**

The experiments described in this report were designed to determine the DNA sequence requirements for ToxT-directed transcription of acfA and acfD, genes that have poorly understood roles in the pathogenesis of *V. cholerae*, but were previously shown to be part of the ToxR regulon (Peterson and Mekalanos, 1988; Parsot and Mekalanos, 1992). Using nested lacZ fusions, we identified the minimal DNA sequences upstream of both acfA and acfD required for ToxT-directed transcription of the respective genes. The minimal ToxT-directed DNA constructs, acfA3 and acfD2, overlap by 52 bp (Figs 1 and 2). This overlap of the DNA sequences required for ToxT-directed transcription of acfA and acfD suggests that there may be a single control region between the two genes utilized by ToxT to activate transcription of both genes.

Mutational analyses of the minimal ToxT-directed DNA constructs for acfA and acfD identified some of the specific sequence requirements for ToxT-directed transcription of these genes. Examination of the DNA sequences upstream of all the genes known to be within the ToxT
The regulon yields no obvious consensus DNA binding sequence. However, tracts of A and T nucleotides are evident upstream of the core promoter elements of each gene. The results of our mutational analyses of some of the A and T-rich sequences between acfA and acfD determined some of the DNA sequence that is specifically required for ToxT-directed transcription of these two genes. Identical mutations in separate acfA::lacZ and acfD::lacZ fusions had similar effects on β-galactosidase expression from both fusions. For example, the acfA16 and acfD11 constructs have A/T to G/C changes to two base pairs at the same positions (Fig. 1). Comparison of β-galactosidase production in strains carrying the acfA16::lacZ and acfD11::lacZ mutant constructs indicates little to no ToxT-dependent transcription from the mutant constructs, strongly suggesting that the base pairs targeted for mutation are required for ToxT-directed transcription of both acfA and acfD. Similarly, the acfA13::lacZ and acfD13::lacZ constructs, which have identical mutations, both allowed no ToxT-directed tran-

Fig. 8. Copper-phenanthroline footprinting of H6-ToxT on acfD constructs having insertions between the two ToxT binding sites. The lanes having DNA cleaved by copper-phenanthroline in the absence of H6-ToxT are indicated by the ‘−’ symbol and the lanes having H6-ToxT/DNA complexes cleaved by copper-phenanthroline are indicated by the ‘+’ symbol. The template construct from which the labelled DNA visible in the gel was amplified by PCR is shown above each pair of lanes. The long arrows represent the putative ToxT binding sites. The brackets beside the bound DNA lanes indicate where insertions between the binding sites are located.

Fig. 9. Results of β-galactosidase assays on strains carrying acfD::lacZ fusions with +5 bp or +10 bp insertions between the ToxT binding sites. The results of experiments performed with V. cholerae strains having wild-type ToxT (O395) are represented as grey bars, and the results of experiments performed with V. cholerae strains having a ToxT deletion (VJ740) are represented as white bars. ‘Allele’ indicates which acfD::lacZ fusion construct was used in the experiment and toxT/toxT indicates the fold difference between the mean β-galactosidase values for that construct measured in O395 and measured in VJ740. β-Galactosidase assays were performed in each strain a minimum of three times, and the values shown are the mean ± standard deviation.
cription. Constructs carrying the identical acfA15 and acfD12 mutations both exhibited reduced, but still significant, levels of activation by ToxT. Again, these results strongly suggest that ToxT is reacting at a single location between acfA and acfD to activate transcription of both genes.

The only mutations that caused significant differences in the degrees of activation by ToxT were the identical acfA17/acfD15 mutations. The acfA17::lacZ construct produced only 2.2-fold higher levels of β-galactosidase in the wild-type toxT strain than in the ΔtoxT strain, whereas the acfD15::lacZ construct produced 8.9-fold higher levels of β-galactosidase in the wild-type toxT strain than in the ΔtoxT strain. The acfA17::lacZ construct did have a considerably higher basal level of transcription than the other acfA::lacZ constructs, which may or may not have any bearing on this comparison. If the β-galactosidase levels of these mutants in the presence of ToxT are compared with the β-galactosidase levels in the wild-type constructs, as the relative percentage of gene expression, the differences are less dramatic. Using this analysis, the acfA17 construct had 44% relative gene expression, and the acfD15 construct had 32% relative gene expression, suggesting that the identical mutations have similar effects on expression of both acfA and acfD. However, it is possible that the base pairs that are mutated in these constructs have different roles in activation of acfA and acfD.

Copper-phenanthroline footprinting studies confirmed that ToxT binds to a single location between acfA and acfD. In these studies, a 28 bp segment of DNA at a central location between acfA and acfD was strongly protected by ToxT, with complete protection seen in the regions of the A/T tracts that are critical for ToxT-directed transcription of both acfA and acfD. The overall region of protection is consistent with our results from the lacZ fusion experiments. The 5′ end points of the lacZ and acfD constructs, from which ToxT is unable to activate transcription, are within the region protected by ToxT in the footprinting experiments. The obvious explanation for this is that ToxT cannot activate transcription from a construct having an incomplete set of DNA binding sites. The 5′ end points of the minimal acfA3 and acfD2 constructs, from which ToxT is able to activate transcription, are well beyond the region protected by ToxT in the footprinting experiments, as would be expected.

Examination of the DNA sequence that shows protection by ToxT in the footprinting experiments and is required for ToxT-directed transcription of acfA and acfD suggests that ToxT binds to two binding sites oriented as an inverted repeat between the two genes (Fig. 6A). This is an unexpected considering that the previously identified ToxT binding sites upstream of ctx and tcpA are most probably oriented as direct repeats (J.H. Withey and V.J. DiRita, in preparation). Moreover, as described above, mutations in either of the putative ToxT binding sites between acfA and acfD, and particularly in the A/T tracts, have similar effects on transcription of both genes. We believe that the 13 bp length of the putative ToxT binding sites is consistent with each of these sites being bound by a single ToxT monomer, based on the lengths of DNA binding sites identified for other proteins in the AraC/XylS family. For example, the related monomeric MarA, SoxS and Rob proteins from Escherichia coli all bind to a degenerate 19 bp DNA sequence (Martin et al., 1999; Griffith and Wolf, 2001), and the dimeric RhaS and AraC proteins each bind to a pair of 17 bp binding sites (Egan and Schleif, 1994; Schleif, 2000). The dimeric AdpA protein from Streptomyces griseus binds to a pair of 10 bp binding sites (Yamazaki et al., 2004). AraC/XylS family proteins have a pair of helix–turn–helix motifs within their DNA binding domains, and thus have the capability to bind to two consecutive major grooves per protein monomer. The lengths of the DNA sequences that are protected by ToxT in footprinting experiments both shown in this report for the acfA–acfD promoter regions and shown previously for the tcpA and ctxA promoter regions (Yu and DiRita, 2002) are most probably too large to be occupied by a single ToxT monomer. The observation that there are two conserved A/T tracts found upstream of acfA, acfD and tcpA (J.H. Withey and V.J. DiRita, in preparation), both of which are required for activation of these genes by ToxT, and the observation that these tracts are in a direct repeat orientation upstream of tcpA and an inverted repeat orientation upstream of acfA and acfD, also make the occupancy of this region by a single ToxT monomer unlikely. Therefore, we propose that one ToxT monomer binds to each of the two binding sites. This idea is further supported by our observation that only a single ToxT binding site is used by ToxT for activation of transcription of aidA (J.H. Withey and V.J. DiRita, in preparation).

Further copper-phenanthroline footprinting studies that assessed the occupancy of the two putative ToxT DNA binding sites when one site carries mutations showed reductions in the protection conferred by ToxT in the region of the mutations. Footprinting experiments using DNA from the acfA16 (actD11) construct, which has a pair of mutations that abrogates ToxT-directed transcription of actA, showed reduced protection by ToxT when compared with the protection conferred by ToxT to DNA from the wild-type acfA3 construct (Fig. 7). However, this reduced protection by ToxT was only evident in the area of the mutations; the unaltered binding site showed no obvious reduction in protection by ToxT. Similarly, experiments using DNA from the acfA17 (actD15) construct, which has a pair of mutations in the 3′ portion of the other putative ToxT binding site that reduces ToxT-directed tran-
scription of acfA and acfD showed reduced protection by ToxT in the region of the mutations. Again, the unaltered binding site showed no obvious reduction in protection by ToxT. This loss of protection is consistent with the idea that occupancy of both binding sites by ToxT, or at least of the regions of the binding sites containing the conserved A/T tracts, is required for activation of transcription by ToxT.

The finding that mutations to one ToxT binding site affect occupancy by ToxT of that site but not occupancy of the unaltered site, together with our observations that ToxT uses a variety of binding site configurations, including inverted repeats, direct repeats and a single binding site, to activate transcription of different genes, raised the question of whether interactions between bound ToxT molecules are required for transcriptional activation. If ToxT is indeed able to bind to pairs of DNA sites having both direct and inverted repeat orientations, as we propose, the protein must be remarkably flexible to form or maintain protein–protein interactions between ToxT monomers in these different orientations. To address this question we altered the spacing between the two acfA/acfD ToxT binding sites by -5 bp and +10 bp and asked whether ToxT would footprint the altered DNA. If ToxT requires interactions between the monomers bound at both sites, or binds to DNA as a dimer, rotating the binding sites one half turn of the helix relative to each other should disrupt any interactions between ToxT molecules and the footprinting of one or both ToxT sites should be affected. Our results strongly suggest that ToxT is able to footprint both binding sites regardless of their positions relative to each other. Therefore, we propose that ToxT binds as independent monomers to the two sites between acfA and acfD.

The consensus DNA sequence for ToxT binding that we have proposed in this work has remarkably little character, other than the presence of a T tract at the 5' end of the site and preference for A/T-rich sequence at the 3' end of the site. This degeneracy is not unusual for AraC/XylS family protein DNA binding sites (Martin et al., 1999; Thomas and Collins, 1999; Ibarra et al., 2003). Studies that have identified the ToxT binding sites upstream of several other genes upon which ToxT acts (J.H. Withey and V.J. DiRita, in preparation) are consistent with the consensus DNA sequence described here. Clearly there must be other contextual cues for ToxT to recognize appropriate binding sites, for a search of only the VPI using this consensus sequence generates dozens of potential ToxT sites, the vast majority of which are not utilized. We propose that the additional contextual information is mediated by interactions between ToxT and RNA polymerase. This may occur by a mechanism known as DNA scanning or pre-recruitment that has been proposed for other AraC family members having degenerate DNA binding sites (Griffith et al., 2002; Martin et al., 2002). According to this model, interaction between the activator protein and RNA polymerase occurs before DNA binding. The extra binding specificity conferred by the RNA polymerase/activator complex permits identification of the appropriate binding sites for that particular activator. In the absence of an adjacent promoter, ToxT would not maintain occupancy of its binding site, and would instead diffuse away with RNA polymerase in search of an appropriate binding site. Previous studies using the tcpA promoter have shown that ToxT most probably interacts with the α-CTD of RNA polymerase (Hulbert and Taylor, 2002; Yu and DiRita, 2002). The spacing of ToxT binding sites described here, relative to the core promoter elements, would also be consistent with ToxT interacting with α-CTD of RNA polymerase as at a class I promoter. Another possibility, provided that ToxT monomers bound to different DNA sites interact with each other, is that only DNA sites with the appropriate spacing(s) and/or orientation(s) can maintain occupancy by ToxT.

Regulation of transcription from divergent promoters by centrally located proteins is quite common in bacteria (Raibaud et al., 1989; Marques et al., 1998; Rhee et al., 1999; Thomas and Collins, 1999; Browning et al., 2002; Recchi et al., 2003). One particularly relevant example is the Mycobacterium tuberculosis Rv1395 activator protein (Recchi et al., 2003), which is also an AraC/XylS family member, although the existence of multiple promoters for both of the divergent genes it regulates, Rv1394c and Rv1395, makes that example considerably more complicated than what we observed for acfA and acfD, each of which has a single promoter. Another AraC family member, UreR, activates transcription of the divergent ureD and ureR genes (D’Orazio et al., 1996; Thomas and Collins, 1999). However, control of these genes is mediated from independent UreR binding sites, so it is unlike what we observed for acfA and acfD, which share a pair of ToxT binding sites. Another well-described protein that can coordinate control of divergently transcribed genes is CRP (El-Robh and Busby, 2002). Divergent promoters that share a central CRP binding site were shown to be independently activated by CRP (El-Robh and Busby, 2002). In that work CRP was acting at class II promoters, at which the regulatory DNA site overlaps the −35 element, for both of the divergently transcribed genes. In contrast, ToxT presumably acts at class I promoters, at which the regulatory DNA site is upstream of the −35 element, to activate both acfA and acfD. Finally, ToxT itself also activates transcription of the divergent aldA and tagA promoters (Parsot and Mekalanos, 1991; Parsot et al., 1991); however, these promoters have independent control regions and there is no overlap between the minimal ToxT-directed aldA and tagA promoter constructs (J.H. Withey and V.J. DiRita, in preparation). Because acfA and acfD...
both encode components of the ACF, it is logical that they would be co-ordinately regulated, whereas aldA and tagA encode an aldehyde dehydrogenase and a putative lipoprotein, respectively, the co-ordinate expression of which may be undesirable.

A previous study on the activation of acfA and acfD transcription indicated that DNA topology is an important factor in the regulation of these genes in V. cholerae (Parsot and Mekalanos, 1992). The results of this previous work suggested that the acfA–acfD region carried on a plasmid in V. cholerae did not show significant transcriptional activation of either gene under inducing conditions, whereas acfA–acfD constructs carried on the bacterial chromosome did show transcriptional activation of both genes under inducing conditions. The most likely explanation for the difference in results between the previous work and our current work is that Parsot and Mekalanos compared expression of their fusions in strains grown under different environmental conditions [37°C, Luria–Broth (LB) at pH 8.4 as non-inducing; 30°C, LB at pH 6.5 as inducing], whereas we compared expression of our fusions in strains having wild-type toxT or a deletion of the region encoding a helix–turn–helix in toxT (VJ740); in all of our experiments the bacteria were grown at 30°C in LB at pH 6.5. Similar experiments we performed in a strain having a complete, in-frame deletion of toxT had results very similar to those obtained using VJ740 (data not shown). Because the experiments described here are geared towards examining only the role of ToxT in activation of acfA and acfD, we believe our experimental system has the fewest variables, and thus is more appropriate for pinpointing ToxT function. However, it is certainly plausible that DNA topology differs when bacteria are grown under different environmental conditions, and this difference in topology could affect gene expression.

To summarize, we have identified the ToxT binding sites located between acfA and acfD required for transcriptional activation of both genes. Two ToxT binding sites arranged in an inverted repeat orientation are located between acfA and acfD and both binding sites are required for ToxT-directed transcription of both acfA and acfD. Mutations to one ToxT binding site do not significantly affect occupancy of the second binding site, and the sizes of the binding sites are consistent with one ToxT monomer binding to each site. DNA insertions between the binding sites did not affect occupancy by ToxT of either site, suggesting that ToxT binds independently to the two sites. However, these insertions did abrogate activation of transcription by ToxT, suggesting that spacing of the binding sites relative to the promoter is an important factor in activation. Both ToxT monomers probably contact RNA polymerase, presumably via the α-CTDs, bound to either the acfA or acfD promoter.

Experimental procedures

Bacterial strains and plasmids

Strains used in this study are listed in Table 1. Strains were grown at 37°C in 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; streptomycin, 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 acfA and acfD nested lacZ fusions were constructed by PCR of the appropriate region using fresh Q395 colonies as template. PCR products were cloned between the XbaI and HindIII sites of pTL61T (Linn and St Pierre, 1990). Site-directed mutations were created using the splicing by overlap extension technique (SOE) (Horton et al., 1989; Horton et al., 1993), after which inserting having the desired mutations were cloned between the XbaI and HindIII sites of pTL61T. 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 (1972).

Copper-phenanthroline footprinting

EMSA was performed as previously described (Yu and DiRita, 2002), except the DNA probe used was a PCR product made using one 3₂P-end-labelled primer and one unlabelled primer. The amount of H₂-ToxT used was determined empirically to be the amount required to shift approximately 50% of the labelled DNA. Plasmids used in the β-galactosidase assays were used as PCR templates as indicated in the text and Table 1. The PCR template used to produce the DNA for footprinting in Fig. 7 was pJW63 (acfA1). After EMSA, the procedure used was that of Papavassiliou (1994). Briefly, the gel was soaked in 200 ml of 10 mM Tris-HCl, pH 8 in a glass tray. One millilitre of 40 mM 1,10-Phenanthroline (Sigma) was mixed with 1 ml of 9 mM CuSO₄ (Sigma) for 1 min, then diluted with 18 ml dH₂O. This was added to the gel tray and
mixed by shaking. Twenty millilitres of a 1:200 dilution of 3-
Mercaptopropionic acid (Sigma) were 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 of dH₂O and placed on X-ray film for 3 h. After
the film was developed, bands corresponding to free DNA
and H₆-ToxT/DNA complexes were excised from the gel
based on their location in the film, and the gel slices were
crushed and the DNA was eluted overnight in 0.5 ml of 0.5 M
Ammonium Acetate, pH 7.5, 1 mM EDTA, 0.1% SDS, 10 mM MgCl₂. 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. Approxi-
mately equal amounts of labelled DNA from the free DNA
and H₆-ToxT/DNA complex bands were loaded on the sub-
sequent sequencing gel. The sequencing ladder was pro-
duced with a Thermo Sequenase Radiolabeled Terminator
Cycle Sequencing Kit (USB) as specified by the manufac-
turer, and the sequencing gel was prepared and run as speci-
fied 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 reac-
tions. Autoradiography was performed with the resulting gel,
and typical exposure times were 10–14 days.

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