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Vibrio cholerae ToxT Independently Activates the Divergently Transcribed *aldA* and *tagA* Genes

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The *Vibrio cholerae* ToxT regulon includes the genes encoding cholera toxin (CT) and the toxin-coregulated pilus (TCP), which are the major virulence factors required for causing cholera disease and colonizing the upper small intestine of the host, respectively. The genes encoding CT, *ctxAB*, and the genes encoding the components of the TCP, *tcpA* to *tcpJ*, are organized within operons, upstream of which are DNA binding sites for the transcriptional activator ToxT. ToxT is a member of the large AraC/XylS family of transcriptional regulators and also activates transcription of five other genes whose roles in *V. cholerae* pathogenesis, if any, are poorly understood. *acfA* and *acfD* are divergently transcribed genes required for efficient colonization of the intestine. Transcriptional activation of *acfA* and *acfD* requires a pair of central ToxT binding sites in an inverted-repeat configuration for ToxT-directed transcription of both genes. *tcpI* has an unknown role in pathogenesis. *aldA* and *tagA* are divergently transcribed genes that also have unknown roles in pathogenesis. In this study, we map the *aldA* and *tagA* promoters and identify the ToxT binding sites upstream of each gene. Our results suggest that two ToxT binding sites in an inverted-repeat configuration are required for ToxT-directed transcription of *tagA* and that a single ToxT binding site is required for ToxT-directed transcription of *aldA*. Furthermore, to direct transcription of *tagA* and *aldA*, ToxT uses independent binding regions upstream of each gene, in contrast to what we previously found for the divergently transcribed *acfA* and *acfD* genes, which share ToxT binding sites between the two genes.

The gram-negative, comma-shaped bacterium *Vibrio cholerae* is the causative agent of the severe diarrheal disease cholera. Cholera continues to be a significant problem in the developing world, where morbidity and mortality levels remain high. There have been seven cholera pandemics since 1817, and the seventh, current pandemic began in 1961 in Indonesia and later spread throughout Africa, South America, and parts of Europe. In 2003 alone, approximately 20,000 people are estimated to have died of cholera worldwide (47).

The two major virulence factors produced by *V. cholerae* are the cholera toxin (CT) and the toxin-coregulated pilus (TCP). Cholera disease is induced by the activity of the cholera toxin, which has an AB_5 stoichiometry (9, 36). The five CT-B subunits are responsible for binding of CT to the host ganglioside-GM1 receptor in the upper small intestine, whereas the single CT-A subunit confers CT activity (18). The CT-A subunit catalyzes ADP-ribosylation of a Gα protein, resulting in elevated levels of cyclic AMP. This then results in secretion of chloride, followed by a massive outpouring of water into the intestinal lumen to produce the voluminous watery diarrhea, also known as rice water stool, characteristic of cholera (7). The genes encoding CT, *ctxAB*, are located within the genome of a lysogenic filamentous phage, CTXΦ (58). The toxin-coregulated pilus, so named because it was originally found to be produced under the same conditions as CT (46, 53, 54), is required for *V. cholerae* to colonize the upper small intestine. TCP is a type IV pilus, and the genes required for TCP biogenesis, *tcpA* to *tcpJ*, are located within a region of *V. cholerae* chromosome I having some phage-like properties, the vibrio pathogenicity island (VPI) (24, 25, 41, 46). TCP is also the receptor for CTXΦ (58).

Expression of *V. cholerae* virulence genes is subject to regulation by a cascade of transcription factors. The direct activator of transcription of the majority of *V. cholerae* virulence genes is the ToxT protein (5), which is a member of the large AraC/XylS family of transcriptional regulators (17). Expression of ToxT is extensively regulated. The membrane-localized transcriptional activators ToxR and TcpP, together with their respective cofactors, ToxS and TcpH, are required to activate transcription of the *toxT* gene by binding to a region upstream of the *toxT* promoter (14, 15, 32, 33). Because *toxT* is also located within the large *tcpA* operon (3, 16), once ToxT protein has been produced it is able to activate its own expression through a positive-feedback loop (61). Expression of the *tcpPH* operon is also subject to regulation by a pair of transcriptional activators, AphA and AphB (26–29, 52). The cyclic AMP receptor protein, CRP, and another protein, PepA, negatively regulate expression of *tcpPH* (2, 29). Finally, in some strains of *V. cholerae* expression of AphA is regulated by the HapR protein, which is controlled by the *V. cholerae* quorum-sensing system (23, 26, 30, 40, 63).

The ToxT protein is 276 amino acids in length, with the 100-amino-acid AraC/XylS family domain at its C-terminal end (17). The AraC/XylS domain contains two helix-turn-helix motifs and confers both DNA binding and transcriptional-activation properties (8, 38, 55). The function of the remaining 176 amino acids of ToxT, which presumably form a second domain, is unknown, and this domain has no homology to any other proteins when a BLAST search is performed with it

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TABLE 1. Strains used in this study

Strain	Plasmid	Relevant genotype	Parent strain	Source or reference
O395		Str^r		Laboratory collection
VJ740		Δ*toxT*	O395	3a
JW21	pTL61T	Amp^r	O395	59
JW22	pTL61T	Amp^r	VJ740	59
JW32	pJW61	*tagA1*::*lacZ*	O395	This work
JW33	pJW62	*tagA2*::*lacZ*	O395	This work
JW38	pJW61	*tagA1*::*lacZ* Δ*toxT*	VJ740	This work
JW39	pJW62	*tagA2*::*lacZ* Δ*toxT*	VJ740	This work
JW42	pJW65	*aldA1*::*lacZ*	O395	This work
JW43	pJW66	*aldA2*::*lacZ*	O395	This work
JW46	pJW65	*aldA1*::*lacZ* Δ*toxT*	VJ740	This work
JW47	pJW66	*aldA2*::*lacZ* Δ*toxT*	VJ740	This work
JW51	pJW70	*tagA3*::*lacZ*	O395	This work
JW55	pJW70	*tagA3*::*lacZ* Δ*toxT*	VJ740	This work
JW65	pJW76	*aldA3*::*lacZ*	O395	This work
JW67	pJW76	*aldA3*::*lacZ* Δ*toxT*	VJ740	This work
JW81	pJW80	*tagA4*::*lacZ*	O395	This work
JW84	pJW80	*tagA4*::*lacZ* Δ*toxT*	VJ740	This work
JW87	pJW82	*aldA4*::*lacZ*	O395	This work
JW92	pJW82	*aldA4*::*lacZ* Δ*toxT*	VJ740	This work
JW97	pJW89	*tagA5*::*lacZ*	O395	This work
JW98	pJW90	*tagA11*::*lacZ*	O395	This work
JW100	pJW92	*aldA11*::*lacZ*	O395	This work
JW101	pJW93	*aldA12*::*lacZ*	O395	This work
JW102	pJW94	*aldA13*::*lacZ*	O395	This work
JW103	pJW95	*aldA14*::*lacZ*	O395	This work
JW105	pJW89	*tagA5*::*lacZ* Δ*toxT*	VJ740	This work
JW106	pJW90	*tagA11*::*lacZ* Δ*toxT*	VJ740	This work
JW108	pJW92	*aldA11*::*lacZ* Δ*toxT*	VJ740	This work
JW109	pJW93	*aldD12*::*lacZ* Δ*toxT*	VJ740	This work
JW110	pJW94	*aldD13*::*lacZ* Δ*toxT*	VJ740	This work
JW114	pJW99	*tagA13*::*lacZ*	O395	This work
JW120	pJW99	*tagA13*::*lacZ* Δ*toxT*	VJ740	This work
JW139	pJW106	*aldA14*::*lacZ*	O395	This work
JW140	pJW107	*tagA14*::*lacZ*	O395	This work
JW145	pJW106	*aldA14*::*lacZ* Δ*toxT*	VJ740	This work
JW146	pJW107	*tagA14*::*lacZ* Δ*toxT*	VJ740	This work
JW155	pJW114	*tagA12*::*lacZ*	O395	This work
JW160	pJW114	*tagA12*::*lacZ* Δ*toxT*	VJ740	This work
JW234	pJW131	*tagA15*::*lacZ*	O395	This work
JW243	pJW131	*tagA15*::*lacZ* Δ*toxT*	VJ740	This work

alone. Possible roles for this second ToxT domain include multimerization of the protein and/or binding to effector molecules. There are no known effectors that are required for transcriptional activation by ToxT, although there is some evidence that bile may act as a negative effector (51).

In addition to its role as the direct activator of *ctx* and *tcpA* transcription, ToxT is known to activate transcription of five other genes whose roles in pathogenesis are poorly understood: *acfA*, *acfD*, *tcpI*, *aldA*, and *tagA* (43–46). All five of these genes are located within the VPI. The *acfA* and *acfD* genes, which are divergently transcribed, encode components of the accessory colonization factor (ACF), which is required for efficient intestinal colonization by *V. cholerae* in the infant mouse model system (46). How these genes function in colonization is unknown; *acfA* encodes a putative outer membrane protein and *acfD* encodes a putative lipoprotein (44, 45). ToxT binds to a pair of binding sites in an inverted-repeat configuration between *acfA* and *acfD* and activates transcription of both genes from this central location (59). *tcpI* encodes a putative methyl-accepting chemotaxis protein (MCP) (13). *tcpI* is divergently transcribed from the *tcpPH* operon, although these operons are relatively distant from one another and are controlled independently. The role of TcpI in pathogenesis, if any, is unknown. *aldA* and *tagA* are also divergently transcribed (43). *aldA* encodes an aldehyde dehydrogenase and *tagA* encodes a putative lipoprotein (12, 43, 45). Again, the roles of AldA and TagA in *V. cholerae* pathogenesis, if any, are unknown.

Previously identified ToxT binding sites upstream of *tcpA*, *ctx*, *acfA*, and *acfD* are consistent with two ToxT monomers binding upstream of each gene (21, 59, 62; J. H. Withey and V. J. DiRita, submitted for publication). AraC/XylS family members are able to bind DNA and activate transcription as both monomers (SoxS, Rob, and MarA) (11, 37) and dimers (AraC and RhaS) (6, 49, 50). The results of experiments on the ToxT binding sites between *acfA* and *acfD*, in which alteration of the spacing between the two sites by the insertion of 5 or 10 bp did not affect the ability of ToxT to footprint both binding sites, strongly suggest that ToxT monomers bind independently to the two binding sites (59).

The sequences of the ToxT binding sites that have been found upstream of *tcpA*, *ctx*, *acfA*, and *acfD* are quite degenerate, which made their identification not a trivial exercise. The

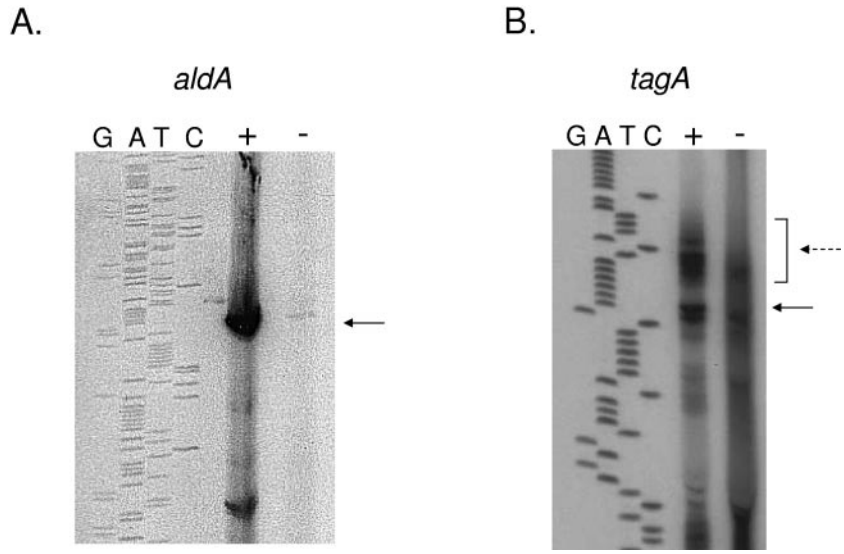

FIG. 1. Primer extensions to determine the start sites of transcription for *tagA* and *aldA*. Primers were designed to hybridize approximately 50 bp downstream of the translational start site. Experiments used total bacterial RNA from *V. cholerae* grown under ToxT-inducing conditions. Arrows indicate the band at the position from which transcription is initiated. The DNA sequencing ladders on the left sides of the figures were produced using the same primers with DNA from wild-type *V. cholerae* colonies as the template. (A) Primer extension of *aldA*. (B) Primer extension of *tagA*. The plus sign indicates that RNA was purified from *V. cholerae* having wild-type *toxT*, and the minus sign indicates that RNA was purified from *V. cholerae* having a *toxT* deletion. The dashed arrow and bracket indicate the bands produced by transcriptional stuttering as described in the text.

major common element in all ToxT binding sites is a tract of four or more T nucleotides on one strand near the 5′ end of the binding site. Mutations to this T tract result in a site to which ToxT may still bind but from which it is unable to activate transcription (59). The 3′ portions of the binding sites have very little sequence conservation but exhibit a preference for A and T nucleotides. Mutations to these 3′ regions generally decrease but do not abrogate activation of transcription by ToxT. In addition to having a degenerate consensus binding sequence, ToxT also uses binding sites oriented in both direct- and inverted-repeat configurations to activate transcription. Upstream of *tcpA*, ToxT binds to a pair of binding sites in a direct-repeat conformation (J. H. Withey and V. J. DiRita, unpublished data); ToxT most likely binds to sites in a direct-repeat orientation upstream of *ctx* as well. However, between *acfA* and *acfD*, ToxT binds to a pair of sites in an inverted-repeat conformation (59). Both of these sites are required for activation by ToxT of both *acfA* and *acfD* transcription. Because the consensus ToxT binding site is so degenerate, it is impossible to identify other ToxT binding sites by a simple sequence search for the consensus. Such a search of only the VPI region of the large *V. cholerae* chromosome yields hundreds of potential ToxT binding sites, the vast majority of which are not used by ToxT.

This study focused on the identification of binding sites from which ToxT activates *tagA* and *aldA*. We located the start sites of transcription and identified the DNA binding sites from which ToxT activates transcription of each gene. Using a combination of nested *lacZ* fusions, directed mutagenesis, and copper-phenanthroline footprinting, we identified ToxT binding sites upstream of both *tagA* and *aldA* from which transcription is activated. Activation of *tagA* transcription by ToxT requires a pair of ToxT binding sites in an inverted-repeat conformation upstream of the core promoter elements. Activation of *aldA* transcription by ToxT requires only a single ToxT binding site upstream of the core promoter elements, an unexpected result given our knowledge of other ToxT-dependent promoters. Although *tagA* and *aldA* are divergently transcribed genes, the minimal DNA sequences from which ToxT activates transcription of *tagA* and *aldA* do not overlap, suggesting that these genes are controlled independently by ToxT.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains used in this study are listed in Table 1. Strains were grown at 37°C in Luria broth (LB) 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 plus 20% glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}, and streptomycin, 100 μg ml^{-1}. Plasmids were introduced into *V. cholerae* strains by electroporation using a Bio-Rad *Escherichia coli* pulser.

DNA manipulations. Plasmids were purified using QIAGEN Spin Miniprep or Plasmid Midi kits. 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 *aldA* and *tagA* nested *lacZ* fusions were constructed by PCR of the appropriate region, using fresh O395 colonies as the template. PCR products were cloned between the XbaI and HindIII sites of pTL61T (34). Site-directed mutations were created using the splicing by overlap extension technique (19, 20), after which inserts 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.

Primer extensions. Transcription start sites were mapped by primer extension as previously described (48). Primers were designed to hybridize approximately 50 bp downstream of the translational start site. Whole-cell RNA was purified using TRIzol reagent (Invitrogen) according to the manufacturer's directions. Bacteria were grown at 30°C for 5 h in LB at pH 6.5 prior to RNA purification. RNA was purified from four strains: for mapping *aldA*, strains JW42 (wild-type [WT] *toxT*, plasmid *aldA*::*lacZ*) and JW46 (Δ*toxT*, plasmid *aldA*::*lacZ*) were used, and for mapping *tagA*, strains JW33 (WT *toxT*, plasmid *tagA*::*lacZ*) and JW46

FIG. 2. Sequence of the *tagA-aldA* intergenic region. Both strands of the DNA are shown, with landmarks for *tagA* on the top strand and landmarks for *aldA* on the bottom strand. The start sites of transcription are shown by an arrow on the top strand for *tagA* and an arrow on the bottom strand for *aldA*. The putative core −35 and −10 elements for each promoter are indicated by boxes. The putative ToxT binding sites upstream of each gene are indicated by arrows between the top- and bottom-strand sequences. The end points of the *tagA3* and *aldA3* constructs are also shown. The positions of mutations are indicated by arrows pointing from the mutant designation to the mutated base pairs. Each of the mutations changes an AT base pair to a GC base pair or vice versa. The bracket below and to the right of the *tagA* start site indicates the poly-T tract on which transcriptional slippage likely occurs. The dots below the sequence indicate 10-bp distances from the start site of *aldA* transcription, and the dots above the sequence indicate 10-bp distances from the start site of *tagA* transcription.

(Δ*toxT*, plasmid *tagA*::*lacZ*) were used (see Fig. 1). Superscript II reverse transcriptase (Invitrogen) was used to elongate primers end labeled with ^{32}P.

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 (39).

Copper-phenanthroline footprinting. An electrophoretic mobility shift assay (EMSA) was performed as previously described (59). The amount of H_6-ToxT (a purified form of ToxT having six histidines added to its N terminus) used was determined empirically to be the amount required to shift approximately 50% of the labeled DNA. Plasmids used in the β-galactosidase assays were used as PCR templates as indicated in the text and Table 1. After EMSA, the procedure used was that of Papavassiliou (42), as previously described (59). Briefly, the gel was soaked in 200 ml 10 mM Tris-HCl, pH 8, in a glass tray. One milliliter 40 mM 1,10-phenanthroline (Sigma) was mixed with 1 ml 9 mM $CuSO_4$ (Sigma) for 1 min and then diluted with 18 ml distilled H_2O. This was added to the gel tray and mixed by shaking. A 1:200 dilution of 3-mercaptopropionic acid (20 ml; 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 1,000 ml distilled H_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 locations in the film. The gel slices were crushed, and the DNA was eluted overnight in 0.5 ml 0.5 M ammonium acetate, pH 7.5, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 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 Tris-EDTA:sequencing stop solution (USB), and radioactivity was measured with a Geiger counter. Approximately equal amounts of labeled 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 labeled in the EMSA/footprinting experiment were used in the sequencing reactions. Autoradiography was performed with the resulting gel, and typical exposure times were 5 to 10 days.

RESULTS

Determination of the start sites of *tagA* and *aldA* transcription. We began our efforts to map the regulatory regions of *tagA* and *aldA* by determining the nucleotides at which transcription is initiated. This was done using primer extension experiments. Oligonucleotide primers were designed to hybridize approximately 50 nucleotides (nt) downstream of the translational start sites of *tagA* and *aldA*. The primer extensions were performed on total cell RNA preparations isolated from bacteria grown under conditions known to induce expression of genes in the ToxT regulon. RNA was isolated from both strains O395 and VJ740 carrying no plasmids and strains JW33 and JW39 (for *tagA*) and JW42 and JW46 (for *aldA*), which carry fusions of the respective gene to *lacZ* on a plasmid, as discussed below. Identical primer extension results were

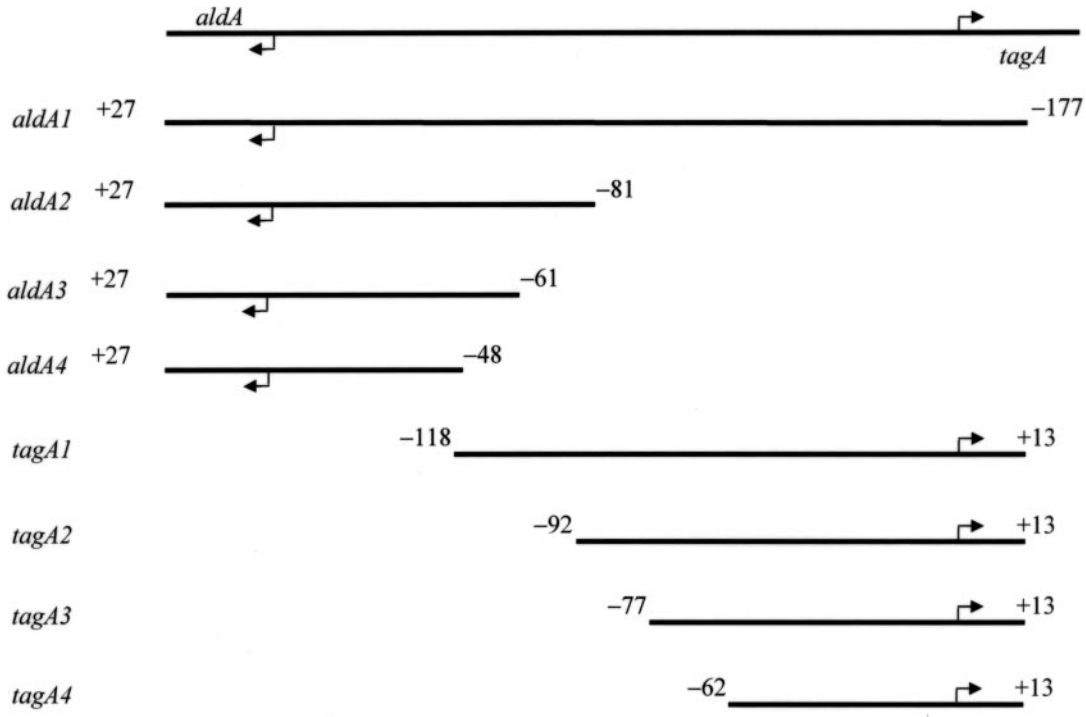

FIG. 3. Map of the nested *lacZ* fusion constructs. The black line at the top of the figure represents the entire *aldA-tagA* intergenic region. Arrows indicate transcriptional start sites. The fusion construct names are listed on the left side, and the size of each construct is represented by the length of the black line. The numbers on either side of the black line indicate the length of DNA present in the construct relative to the transcriptional start site.

observed from mRNAs produced from either the chromosomal copy (data not shown) or the plasmid copy of either gene, although the bands produced using the plasmid-based genes were significantly more intense (Fig. 1). A single band was observed using primers for *aldA* (Fig. 1A). However, multiple bands were observed using primers for *tagA* (Fig. 1B). This may be due to RNA polymerase (RNAP) slippage or stuttering at a tract of T nucleotides on the template strand 4 nt downstream from the site we propose as the primary *tagA* transcriptional start (Fig. 2). Transcriptional slippage would lead to insertion of a random number of additional A nucleotides before RNAP resumes normal elongation, resulting in the ladder of bands observed just above the band corresponding to the start site in Fig. 1B. Stuttering or transcriptional slippage, which leads to incorporation of nontemplated nucleotide tracts within the mRNA, has been observed in several other instances when there is a mononucleotide tract in the template strand at or near the start site of transcription (1, 4, 10, 22, 35, 56, 57, 60). Because the added nucleotides in this case would be upstream of the *tagA* translational start site, stuttering would affect neither translation nor the reading frame of *tagA*. Our assignment of the true *tagA* start site is based primarily on the observation that a near-consensus −10 element is present at the appropriate position relative only to this nucleotide and on the fact that the band in Fig. 1B corresponding to this nucleotide is the highest in the gel aside from the stuttering ladder above it. Our goal with the series of experiments described here was to map the ToxT binding sites responsible for activating *tagA*, so the apparent transcriptional slippage phenomenon we observed was not examined further.

The putative core promoter −10 and −35 elements were identified based on the locations of the start sites of transcription for *aldA* and as noted above for *tagA* (Fig. 2). There is a near-consensus −10 box (TATAAT) found upstream of both genes. However, both genes have a degenerate putative −35 box (TTGACA) at the appropriate spacing relative to the −10 element. We previously identified degenerate −35 boxes upstream of *acfA*, *acfD*, and *tcpA* (59, 62), suggesting that this may be common among genes in the ToxT regulon.

Identification of the minimal *tagA* promoter region. To determine the length of DNA upstream of the core promoter elements of *tagA* that is required for ToxT-directed transcription, we constructed nested *tagA*::*lacZ* fusions in which the 5′ endpoint of DNA upstream from *tagA* varies from −118 to −62 relative to the start site of transcription (Fig. 3). These fusions were constructed in plasmid pTL61T, which carries a promoterless *lacZ* gene downstream from multiple restriction sites. The ability of ToxT to activate transcription of these *tagA*::*lacZ* constructs was assessed by measuring β-galactosidase activity in *V. cholerae* strains having either wild-type *toxT* or a *toxT* deletion. Constructs extending to −118, −92, and −77 exhibited similar levels of ToxT-directed transcription (Fig. 4). However, a construct extending to −62 did not exhibit significant ToxT-directed transcription. This suggests that the sequence between −77 and −62 contains sequence elements that are essential for activation of *tagA* transcription by ToxT.

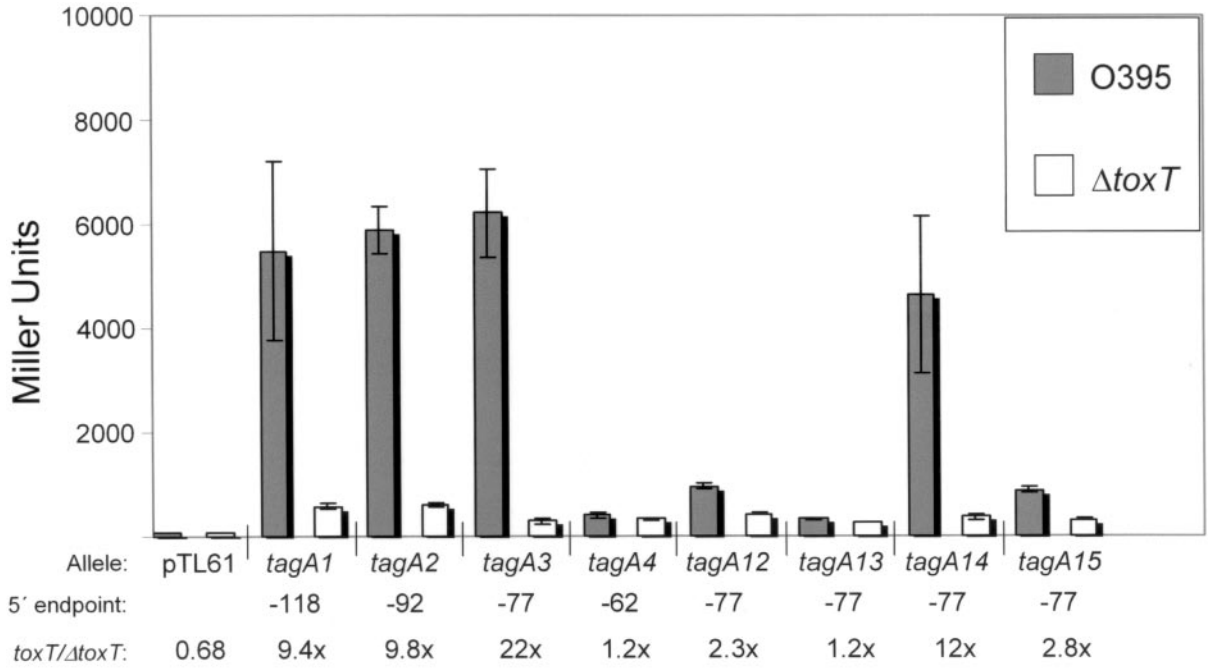

FIG. 4. Results of β-galactosidase assays with strains carrying *tagA*::*lacZ* fusions. The results of experiments done with *V. cholerae* strains having wild-type ToxT (O395) are represented as gray bars, and the results of experiments done with *V. cholerae* strains having a ToxT deletion (VJ740) are represented as white bars. "Allele" indicates which *tagA*::*lacZ* fusion construct was used in the experiment, except for pTL61T, in which the empty vector was present in the indicated strains; "5′ endpoint" indicates the length of DNA upstream of the transcriptional start site that is present in the construct; and "*toxT*/Δ*toxT*" indicates the difference (*n*-fold) between the mean β-galactosidase values for that construct measured with O395 and measured with VJ740. β-Galactosidase assays were performed a minimum of three times with each strain, and the values shown are the means ± standard deviations.

Mutational analysis of the *tagA* promoter region. Previous work suggested that ToxT binds to a 13-bp DNA sequence having a tract of 4 or more consecutive T nucleotides on one strand, followed by a sequence rich in A and T nucleotides (59). The region upstream of *tagA* between the −35 box and −77, the 5′ endpoint of the minimal ToxT-activated *tagA3* construct, contains two T tracts that would be consistent with this ToxT consensus sequence. We targeted these tracts for mutagenesis, along with some other A/T-rich sequences in this region, to examine the DNA sequence requirements for ToxT-directed transcription of *tagA*. Site-directed mutations were created in the *tagA3* construct, which extends to −77 relative to the start of transcription. Each of the mutations changes an AT base pair to a GC base pair.

Mutations to the base pairs at −49 and −51 (*tagA13*), in the midst of a tract of 7 T nucleotides (Fig. 2, bottom strand), caused the largest defect in ToxT-directed transcription of *tagA* (Fig. 4). The *tagA13* construct showed no significant difference in β-galactosidase activity between strains having wild-type *toxT* and strains having a *toxT* deletion. Mutations to the base pairs at −75 and −76 (*tagA12*), in the midst of a tract of 4 T nucleotides (Fig. 2, top strand), also caused a large defect in ToxT-directed transcription, reducing the difference in β-galactosidase activity between *toxT* and Δ*toxT* strains to 2.3-fold, versus the 22-fold difference seen with the parent *tagA3* construct (Fig. 4). These results are consistent with a requirement for two ToxT binding sites upstream of *tagA* for transcriptional activation. The observation that the T tracts in these two putative binding sites are on opposite strands of the DNA suggests that these sites are arranged in an inverted configuration, similar to what we found previously between *acfA* and *acfD* (59). Mutations in the 3′ portion of the promoter-distal ToxT activation site upstream of *tagA*, at −69 and −70 (*tagA15*), caused a large reduction in the ability of ToxT to activate transcription. However, mutations located between the two putative ToxT binding sites (Fig. 2), at −63 and −64 (*tagA14*), had no significant effect on ToxT-directed transcription of *tagA*.

Identification of the minimal *aldA* promoter region. We used a method similar to that described above for *tagA* to determine the length of DNA upstream of the core promoter elements of *aldA* that is required for ToxT-directed transcription. Nested *lacZ* fusions were constructed in pTL61T, and β-galactosidase levels for strains carrying these fusions and having either wild-type *toxT* or a deletion in *toxT* were assayed. This series extended from −177 bp upstream to −48 bp upstream of the transcription start site (Fig. 3). Compared to the levels of induction of other ToxT-activated genes, those conferred by ToxT to the *aldA*::*lacZ* fusions were relatively low. Constructs extending to −177, −81, and −61 all had similar levels of expression in the wild-type *toxT* background, with values in the range of 1,200 to 1,600 Miller units (Fig. 5). For other ToxT-activated genes, the range is from 5,000 to over 20,000 Miller units. Significantly, however, the *aldA*::*lacZ* construct extending to −48 expressed β-galactosidase at levels markedly lower than those expressed in the longer constructs and exhibited little to no ToxT-directed transcription.

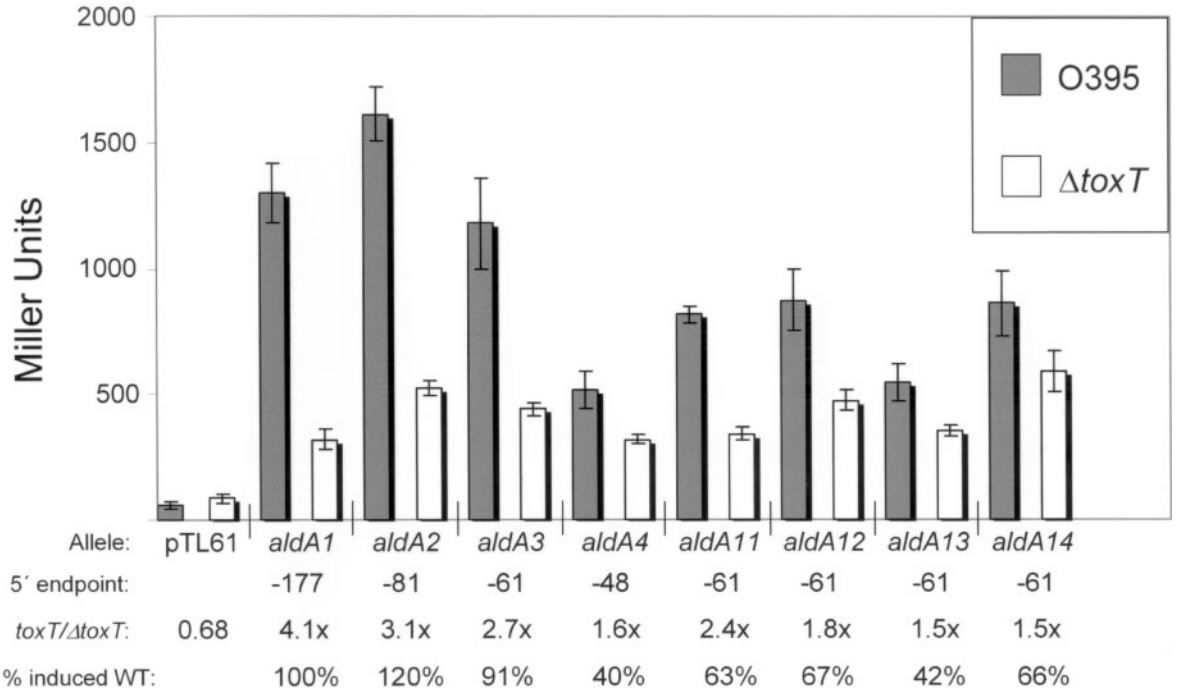

FIG. 5. Results of β-galactosidase assays with strains carrying *aldA*::*lacZ* fusions. The results of experiments done with *V. cholerae* strains having wild-type ToxT (O395) are represented as gray bars, and the results of experiments done with *V. cholerae* strains having a ToxT deletion (VJ740) are represented as white bars. "Allele," "5′ endpoint," and "*toxT*/Δ*toxT*" are defined in the legend for Fig. 4. "% induced WT" was calculated by dividing the mean β-galactosidase level for the indicated construct by the mean β-galactosidase level from the *aldA1* construct which contains the entire *tagA-aldA* intergenic region. β-Galactosidase assays were performed a minimum of three times with each strain, and the values shown are the means ± standard deviations.

Comparison of these nested constructs in terms of the level of induction (*n*-fold) by ToxT, as we have done previously, is complicated by the observation that the basal levels of expression in the Δ*toxT* background varied between different *aldA*::*lacZ* fusion constructs. While this is commonly observed for other *lacZ* fusion constructs as well, including those we describe here for *tagA*, because the relative levels of expression of the *aldA*::*lacZ* constructs in wild-type *toxT* strains are lower than those of *tagA*::*lacZ* constructs, small differences in measurements of basal activity of the *aldA*::*lacZ* constructs in the Δ*toxT* strains have a large effect on the calculation of induction due to ToxT activity. For example, the −61 construct (*aldA3*) produced 91% of the β-galactosidase activity of the longest (−177) construct (*aldA1*) in strains having wild-type *toxT*. However, the differences (*n*-fold) between the wild-type *toxT* and Δ*toxT* strains carrying these constructs were 2.7-fold and 4.1-fold, respectively. Therefore, we believe it is most useful to assess these constructs as percentages of the ToxT-induced expression observed for the full-length, −177 construct (*aldA1*) (Fig. 5). Using this assessment, it is clear that the −48 construct, *aldA4*, has a defect in ToxT-directed transcription, whereas the −177, −81, and −61 constructs have similar levels of expression. This suggests that the DNA sequence between −48 and −61 is important for ToxT-directed transcription of *aldA*.

Mutational analysis of the *aldA* promoter region. We targeted A/T-rich sequences upstream of *aldA* for mutagenesis to determine whether any of these sequences are important for ToxT-directed transcription of *aldA*. Mutations were constructed in the minimal ToxT-directed *aldA*::*lacZ* construct, *aldA3*, which extends to −61 relative to the start of transcription. Each of the mutations changes an AT base pair to a GC base pair or vice versa.

The sequence between the −35 box and −61 upstream of *aldA* has three tracts of 3 or more consecutive T nucleotides, and we mutated each of these. Mutations at −52 and −54 (*aldA13*) had the strongest negative effect, resulting in a ToxT-directed transcription level of only 42% of the wild-type level. This is very similar to the level seen for the truncated construct extending to −48 (*aldA4*). The other mutations each resulted in a minimal decrease in ToxT-directed transcription to around 65% of that observed with the wild-type −177 construct. The observations that (i) the length of the minimal *aldA*::*lacZ* fusion exhibiting ToxT-directed transcription is much shorter than that which we have observed for other genes in the ToxT regulon and (ii) that mutations to only one T tract caused a drastic decrease in the ability of ToxT to activate transcription of *aldA* suggest that a single ToxT binding site may be located upstream of *aldA*, in contrast to the other ToxT-activated operons we have studied.

Copper-phenanthroline footprinting of the *aldA-tagA* intergenic region. The genetic experiments described above allowed us to determine regions between *tagA* and *aldA* that are required for ToxT-directed transcription. To more precisely identify the DNA sequence bound by ToxT, we used DNA footprinting. The copper-1,10-phenanthroline footprinting procedure was used because of its superior resolution and relative

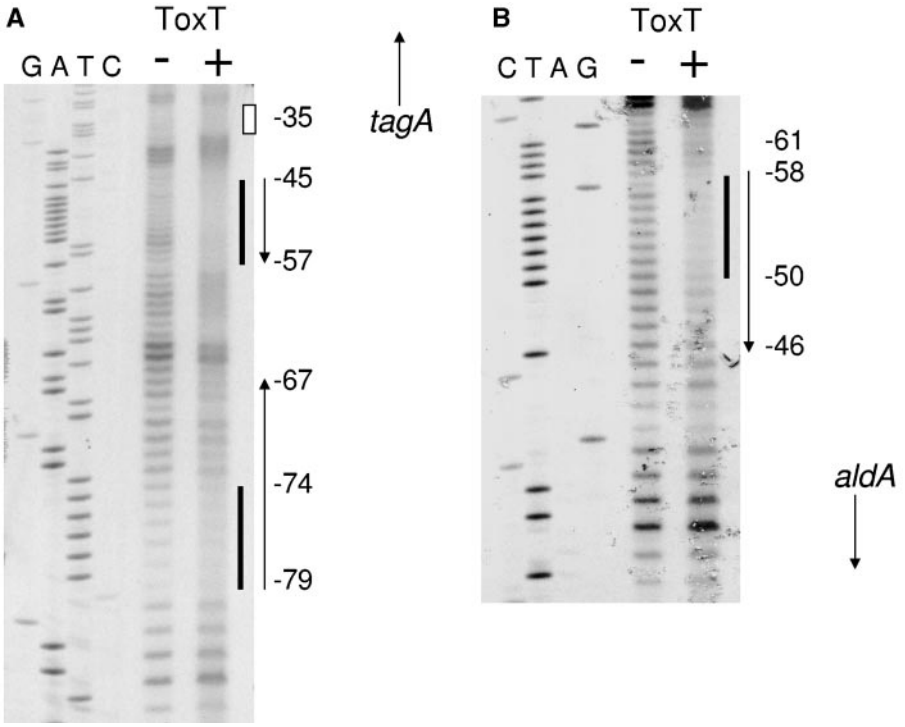

FIG. 6. Copper-phenanthroline footprint of H_6-ToxT in the *tagA-aldD* intergenic region. −, DNA cleaved by copper-phenanthroline in the absence of H_6-ToxT; +, H_6-ToxT/DNA complexes cleaved by copper-phenanthroline. The numbers to the right of the autoradiographs indicate the distances upstream of the transcriptional start sites of *tagA* and *aldD*. The −35 core promoter elements are represented by empty boxes. (A) Footprinting of H_6-ToxT on the minimal *tagA3* construct. The vertical arrows indicate the positions of the putative binding sites, and the heavy black lines indicate the regions of protection. (B) Footprinting of H_6-ToxT proximal to *aldA* alone. The vertical arrow indicates the position of the binding site, and the heavy black line indicates the region of protection.

lack of sequence and structural cleavage specificity relative to DNase I (42, 59). We used a purified form of ToxT having six histidines added to its N terminus (H_6-ToxT) for these experiments; previous work showed that H_6-ToxT is functional both in vivo and in vitro (59, 62).

Three regions of protection were conferred by H_6-ToxT within the *tagA-aldA* intergenic region. The first and second of these protected regions were proximal to *tagA* (Fig. 6A); DNA segments between −45 and −57 and between −79 and −74 relative to the start of *tagA* transcription were protected by H_6-ToxT. These footprints encompass both of the T tracts to which directed mutagenesis produced the strongest negative effect on ToxT-directed transcription of *tagA*. The third protected region encompassed the T tract between −50 and −56 relative to the start of *aldA* transcription (Fig. 6B). Again, this was the region to which directed mutagenesis produced the strongest negative effect on ToxT-directed transcription of *aldA*. The sequence between the putative *tagA* and *aldA* ToxT binding sites was not significantly protected by H_6-ToxT (not shown).

Our assignment of the binding-site sequences (indicated by the arrows in Fig. 2 and 6) is based on both the genetic data described in this report and our studies of ToxT binding sites upstream of *acfA*, *acfD*, and *tcpA* that indicate ToxT binds to a 13-bp sequence with a T tract near the 5′ end (59; J. H. Withey and V. J. DiRita, submitted). The 5′ regions of both *tagA* binding sites, containing the T tracts, exhibited the highest levels of protection. However, there is a significant disparity in the degrees of protection conferred by H_6-ToxT to the *tagA*-proximal and *tagA*-distal binding sites, particularly in the 3′ portions of the binding sites; the *tagA*-proximal binding site is strongly protected along its length, whereas the *tagA*-distal binding site is more weakly protected within the T tract, and little to no protection is observed for the 3′ portion of this binding site. This result is consistent with our observation that mutations to the T tract in the *tagA*-proximal binding site at −55 to −49 resulted in a complete loss of activation by ToxT (*tagA13* mutant [Fig. 4]), whereas mutations to the T tract in the *tagA*-distal binding site at −76 to −79 resulted in a large decrease, but not a complete loss, of activation by ToxT (*tagA12* mutant [Fig. 4]). Significant protection by H_6-ToxT was not observed for the sequence between the two *tagA* ToxT binding sites in this experiment.

DISCUSSION

The experiments in this report were designed to identify the DNA binding sites from which ToxT activates transcription of the divergent *tagA* and *aldA* genes, which were previously shown to be within the ToxT regulon (5, 12, 43, 45, 46) but which have unknown roles, if any, in *V. cholerae* pathogenesis. Earlier work by Parsot and Mekalanos (43) had roughly mapped the regulatory region for *tagA* to a region within about

Gene:	Sequence:	Repeat:	Spacing:	Promoter Proximity:
tagA:	AATTTTAAGTTAA TGTTTTTTTAATG	Inverted	9bp	-45
aldA:	TGTTTTTTTAAAT	Single	NA	-45
acfA/acfD:	AATTTTTAAAAAT CATTTTGTTAAAT	Inverted	2bp	-51/-46
tcpA:	TATTTTTTTAATA CATTTTTTGCTGT	Direct	7bp	-45
ctxAB:	GATTTTTGATTTT GATTTTTGATTTT	Direct	8bp	-80
Consensus:	xATTTTTWWWWWW			

FIG. 7. Alignment of ToxT binding sites. "Gene" indicates the locus upstream of which the ToxT binding sites were identified. "Sequence" indicates the sequence of the ToxT binding site. "Repeat" indicates the orientation of the binding sites relative to each other except for the *aldA* binding site. "Spacing" indicates the number of base pairs between the two ToxT binding sites, except for *aldA*, for which "NA" indicates not applicable. "Promoter proximity" indicates the promoter-proximal end of the nearest ToxT binding site relative to the start site of transcription. "W" in the consensus sequence represents either an A or a T nucleotide.

400 bp upstream of the translational start site. Using nested *lacZ* fusions, we identified the minimal DNA sequences upstream of both *tagA* and *aldA* required for ToxT-directed transcription of the respective genes. Unlike what we previously found for the similarly divergent ToxT-activated genes *acfA* and *acfD*, the minimal ToxT-directed *tagA* and *aldA* constructs do not overlap (Fig. 3); there are 24 bp between the endpoints of *tagA3* and *aldA3*, the minimal constructs for each gene that permit ToxT-directed transcription (Fig. 2). This finding suggests that *tagA* and *aldA* have independent ToxT binding regions from which transcription is activated.

Mutational analysis of the A/T-rich sequences upstream of *tagA* gave us further information about the DNA sequence requirements for its ToxT-directed transcription. Two T tracts upstream of *tagA* are required for ToxT-directed transcription of *tagA*. Mutations in the promoter-proximal T tract (*tagA13*) completely abrogated transcriptional activation by ToxT, whereas mutations in the promoter-distal T tract (*tagA12*) drastically reduced transcriptional activation by ToxT. The observation that these two T tracts are on opposite DNA strands suggests that there are two putative ToxT binding sites upstream of *tagA* arranged as an inverted repeat. This is similar to what we observed for the ToxT sites between *acfA* and *acfD* (59). A notable difference between the *tagA* ToxT sites and the *acfA* to *acfD* ToxT sites is their spacing relative to each other; there are 2 bp between the two *acfA* to *acfD* ToxT sites and 9 bp between the two *tagA* ToxT sites (Fig. 2). One likely explanation for this difference is that the two ToxT sites located between *acfA* and *acfD* are used to activate transcription of both genes (59), whereas the two ToxT sites upstream of *tagA* are used only to activate *tagA* transcription. Therefore, there are likely different requirements for optimal spacing of the sites for each individual site and relative to the core promoter elements in these different situations.

Copper-phenanthroline footprinting of H_6-ToxT in the *tagA* region further localized the ToxT binding sites upstream of *tagA*. The two T tracts we identified as most critical for transcriptional activation by ToxT were in the regions of greatest protection by H_6-ToxT. However, greater protection was conferred by H_6-ToxT to the *tagA*-proximal binding site than to the *tagA*-distal binding site (Fig. 6A). This finding is consistent with the results of the *lacZ* fusion experiments with mutated binding sites, which indicated that mutagenesis to the *tagA*-proximal binding site had a larger negative effect on ToxT-directed transcription of *tagA* than did mutagenesis to the *tagA*-distal binding site. Perhaps binding by ToxT to the *tagA*-distal binding site is more dependent on nonspecific interactions with the phosphate DNA backbone or the likely interaction between ToxT and the α-C-terminal domain of RNAP provides more binding energy at this position.

Mutational analysis of the A/T-rich sequences upstream of *aldA* identified a 7-nt T tract as important for ToxT-directed transcription of *aldA*. The results of the *aldA* experiments are significantly more difficult to interpret due to the relatively low level of induction conferred by ToxT even from a construct carrying the entire *tagA-aldA* intergenic region. This level of induction is low only compared to other ToxT-activated promoters, however; over 1,200 Miller units of β-galactosidase is considerable activity. Because the levels of expression of the truncated *aldA4* construct, which presumably lacks a ToxT binding site, and the mutated *aldA13* construct, which has a pair of mutations in the 7-nt T tract, are very similar, it is safe to say that the *aldA13* mutations cause as large a defect in ToxT-directed transcription as we are likely to see using this promoter construct. The other *aldA*::*lacZ* mutant constructs decreased but did not abolish ToxT-directed transcription of *aldA*. This is similar to what we have observed for mutations in other ToxT binding sites outside of the conserved T tract. We also observed protection conferred by H_6-ToxT to the 7-nt *aldA* T tract in footprinting experiments (Fig. 6B), confirming

the position of ToxT in the *aldA* promoter region, but did not observe significant protection outside of the T tract.

The most notable difference between the region upstream of *aldA* that we found to be required for ToxT-directed transcription and the regions upstream of other operons in the ToxT regulon that we have studied is the relative shortness of the minimal *aldA* DNA sequence from which ToxT-directed transcription occurs (*aldA3* [Fig. 2 and 3]). Based on our observations of the requirements for ToxT-directed transcription of *acfA*, *acfD* (59), *tagA*, *tcpA* (J. H. Withey and V. J. DiRita, submitted), and *tcpI* (J. H. Withey and V. J. DiRita, submitted), there is not sufficient DNA sequence between the −61 endpoint of the *ald3* construct and the −35 box in which to fit two ToxT binding sites. Furthermore, there is only a single T tract at an appropriate position in this region to which mutagenesis produces a dramatic decrease in ToxT-directed transcription. Therefore, we propose that there is a single ToxT binding site upstream of *aldA*. This would be the first ToxT-activated gene that has been found to use only one binding site; this also suggests that ToxT acts as a monomer at *aldA*. We have previously observed that ToxT binds to DNA as monomers to both of the binding sites between *acfA* and *acfD* (59) and to both of the binding sites upstream of *tcpA* (J. H. Withey and V. J. DiRita, submitted). The putative single ToxT site at *aldA* is however different in its orientation relative to the promoter from other promoter-proximal ToxT binding sites we have identified. The promoter-proximal binding sites at the *acfA*, *acfD*, *tagA*, *tcpA*, and *tcpI* promoters are all oriented so that the conserved T tract is on the template strand, and thus the binding site "points away" from the promoter (Fig. 2). The *aldA* ToxT site has the conserved T tract on the nontemplate DNA strand, and so the binding site "points toward" the promoter (Fig. 2). However, the *aldA* ToxT site is located at a position similar to the promoter-proximal ToxT sites of the other genes, and the sequence of the *aldA* ToxT site fits with the consensus ToxT binding sequence we have proposed based on ToxT sites upstream of other genes (Fig. 7). The difference in orientation could potentially explain why the level of *aldA* induction conferred by ToxT is low relative to the level of induction conferred by ToxT at other promoters.

Our observation that the divergent *aldA* and *tagA* genes are controlled independently by ToxT adds to the question of what the roles these genes have as members of the ToxT regulon. There are several operons within the VPI that are oriented divergently, including *acfA-acfD* and *tcpI-tcpPH*. As we have shown previously, transcription of *acfA* and *acfD* is activated by ToxT from a central location between the genes (59), and presumably *acfA* and *acfD* expression levels are coregulated. Because both of these genes encode components of ACF, it is logical that their expression would be coordinated. *tcpI* and *tcpPH*, on the other hand, have separate control regions and a relatively large intergenic distance, and their transcription levels are controlled by different transcriptional regulators (2, 26–29, 31, 52). Because TcpP and TcpH are required for expression of *toxT* and ToxT activates expression of *tcpI*, these genes would have no obvious reason to be coordinately regulated. This suggests that the independent regulation by ToxT of *aldA* and *tagA* may be due to TagA and AldA proteins having different roles in either cell physiology or pathogenesis and that their expression levels may differ under different ToxT levels. Because the ToxT-directed expression of *aldA* is relatively low and *aldA* uses only a single ToxT binding site, high concentrations of ToxT may be required for full induction of *aldA*. Conversely, because *tagA* is activated to a relatively high degree by ToxT and uses two ToxT binding sites, lower concentrations of ToxT may allow full induction of *tagA*. Further work on the roles of these gene products in *V. cholerae* biology should address whether this hypothesis is sound.

In summary, we have identified the ToxT binding sites upstream of *tagA* and *aldA* from which ToxT activates transcription of these genes. *tagA* utilizes two ToxT binding sites in an inverted-repeat conformation, whereas *aldA* utilizes a single ToxT binding site. The sequences of these newly identified binding sites are in accordance with the ToxT consensus binding sequence we have previously proposed (59). The minimal constructs from which ToxT is able to activate *tagA* and *aldA* transcription do not overlap, suggesting that ToxT controls expression of *tagA* and *aldA* independently.

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