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# The Virulence Gene Activator ToxT from *Vibrio cholerae* Is a Member of the AraC Family of Transcriptional Activators

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**Virulence gene expression in *Vibrio cholerae* is postulated to involve ToxR-dependent activation of the *toxT* gene followed by ToxT activation of virulence genes, including several of those involved in biogenesis of the toxin-coregulated pilus. ToxR is a transmembrane, DNA-binding protein which is a member of the OmpR subclass of two-component activator systems in bacteria. Data presented in this report demonstrate that ToxT is similar to the AraC family of transcriptional activators identified in a variety of gram-negative bacteria. The *toxT* open reading frame begins approximately 200 nucleotides from the end of the *tcpF* gene, which is part of a cluster of genes responsible for production of the toxin-coregulated pilus. Accumulation of *toxT* specific mRNA is ToxR dependent and is modulated by environmental conditions that modulate expression of the regulon. Within the intergenic region between *tcpF* and *toxT* is a potential stem-loop structure of an unusual nature which may play a role in regulating expression of *toxT* mRNA. Experiments with *tcpF* and *toxT* cloned behind a strong, constitutive promoter suggest that the two genes can be cotranscribed, but Northern (RNA) blot analysis of *V. cholerae* suggests that if they are, steady-state levels of their messages may be controlled by a posttranscriptional mechanism. Possible mechanisms for ToxR-dependent expression of *toxT* are discussed.**

Regulation of several genes required for virulence of the human diarrheal pathogen *Vibrio cholerae* is under general control of two proteins, ToxR and ToxS (12, 13, 15, 18, 23). ToxR is an atypical member of the OmpR subclass of two-component regulators in bacteria, in that it does not have the phosphoacceptor domain homology characteristic of this family and thus must become activated in some way other than by being phosphorylated by a sensor kinase (15, 21). It has been proposed that ToxR may function as a homodimer and that ToxS is required for the stability of such dimers (5). Another aspect of ToxR that makes it different from the other members of the family is that although it is a transcriptional activator, it is also a membrane protein that binds the promoter for the operon encoding cholera toxin (*ctxAB*) while associated with the membrane (15).

Other ToxR-regulated genes are controlled indirectly by it, in that ToxR is required for them to be expressed but it does not directly activate their transcription. These include several genes required for assembly of the toxin-coregulated pilus (TCP) (23), a major colonization factor, and several accessory colonization factor (ACF) genes (18). The observation that ToxR is not sufficient for expression of virulence genes other than *ctxAB* led to identification of the *toxT* gene from *V. cholerae*. *toxT* encodes a product that can directly activate many ToxR-regulated genes, including *tcpA*, *tcpC*, *tcpI*, and *ctxAB*, and is itself under ToxR control (6). In this report, we demonstrate that the *toxT* product is related to the AraC family of transcriptional regulator proteins and demonstrate that *toxT* is located within the *tcp* gene cluster downstream of the *tcpF* gene and upstream of the *tcpJ* gene. Preliminary transcription analysis of *toxT* is also presented.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Escherichia coli* VM2 was used to assess ToxT activity. This strain is lysogenic for λ carrying a *ctx-lacZ* gene fusion and has been described previously (13). Plasmid pGJ40 is a pBR327 derivative isolated from a plasmid library of *V. cholerae* 569B and was previously shown to encode an activator of several ToxR-regulated genes (6). Various derivatives of pGJ40 described herein were generated to determine the approximate location of the *toxT* gene within the cloned *V. cholerae* DNA. Bacterial strains were maintained at −70°C in Luria-Bertani medium plus 20% glycerol.

**DNA manipulations.** Plasmid DNA was purified by using QIAGEN columns (QIAGEN, Inc.). Cloning and mutagenesis procedures were done by using standard protocols (11). A double-stranded-DNA sequencing protocol (kindly supplied by Yan Su) with synthesized sequencing primers was used. Plasmid DNA (5 μg) was dried under a vacuum, resuspended in 2 μl of 0.2 N NaOH, and incubated at 25°C for 2 to 3 min. Primer (2 μl; approximately 500 to 1,000 ng) was added, and then 2 μl of double-distilled water and 2 μl of annealing buffer (833 mM Tris-HCl [pH 7.5], 83 mM $MgCl_2$) were added and the annealing was allowed to proceed for 2 min at 25°C, at which time 2 μl of 0.2 N HCl was added. Primer template hybrids prepared in this way were subjected to DNA sequence analysis with the Sequenase version 2.0 kit (U.S. Biochemical Co., Cleveland, Ohio). Sequencing products were resolved on 6.0 and 8.0% gels which were fixed, dried, and exposed to Kodak X-Omat film overnight at room temperature.

**RNA analysis.** RNA was isolated from overnight cultures of *V. cholerae* by the hot-phenol method previously described (6). RNA (10 μg per lane) was resolved on 1.3% agarose–6% formaldehyde gels in MOPS (morpholine propanesulfonic acid) buffer (11). A separate set of lanes was always loaded and cut off from the remainder of the gel prior to blotting. This was stained with 0.5 μg of ethidium bromide per ml in 0.1 M ammonium acetate (11) in order to ensure that equivalent amounts of RNA were loaded on the gels. The unstained portions of the gels were blotted overnight to nitrocellulose (Gelman Scientific, Ann Arbor, Mich.) with-

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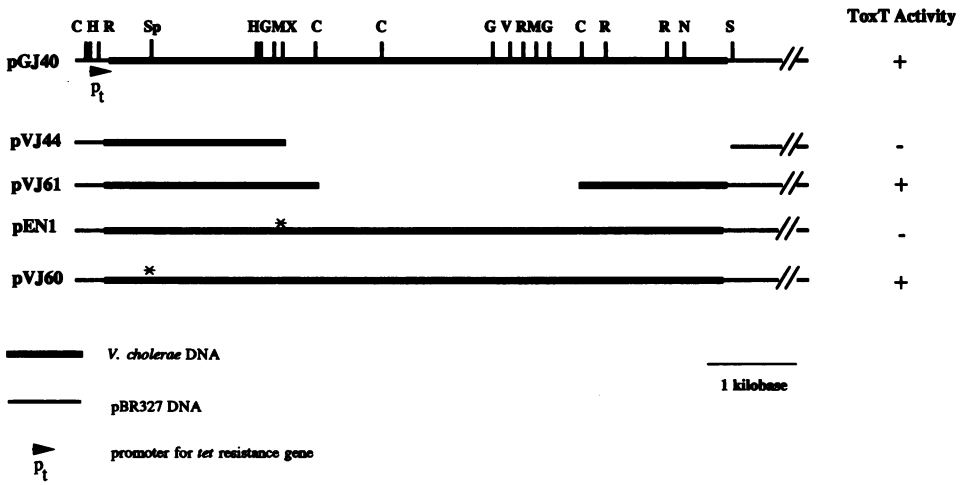


FIG. 1. Map of pGJ40. The plasmid harbors approximately 7 kbp from *V. cholerae* 569B cloned as a *Sau*3A fragment into the *Bam*HI site of pBR327. ToxT activity is defined as the ability to activate a *ctx-lacZ* fusion gene in *E. coli*. Mutant derivatives are depicted below pGJ40. Deletions are represented by gaps; asterisks represent the location of frameshift mutations generated by restriction enzyme digestion (*Spe*I in pVJ60 and *Xho*I in pVJ44). Restriction enzyme sites are as follows: C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; M, *Mlu*I; N, *Nco*I; R, *Eco*RV; S, *Sal*I; Sp, *Spe*I; X, *Xho*I.

out prior treatment. Blots were baked in the morning in a vacuum oven at 80°C. Northern (RNA) analysis was done with restriction fragments removed from agarose gels (GTG agarose; FMC, Rockland, Maine) and labelled with $^{32}P$ by using a nick translation kit (Amersham, Arlington Heights, Ill.). For *tcpEF*, the probe used was an *Xho*I-*Spe*I fragment from plasmid pDH8 which contains the 3′ end of the *tcpE* gene and the 5′ end of *tcpF*. The *toxT* probe used was the *Hin*dIII-*Xho*I fragment fully within *toxT* shown in Fig. 1. Blots were prehybridized for 4 h at 42°C in 50% formamide–5× Denhardt's solution–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 8.0])–0.1% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml. Blots were hybridized overnight at 42°C in the same solution except for the replacement of 5× Denhardt's solution with 2× Denhardt's solution. Blots were given two 10-min washes both at 25°C in 2× SSPE–0.1% SDS and 0.2× SSPE–0.1% SDS. After being washed, the blots were dried and exposed to Kodak X-Omat film at −70°C with intensifying screens (Cronex Lightning; Dupont).

**Isolation of the O395 *toxT* locus.** Plaques from a λ-GEM11 library of *V. cholerae* O395 DNA (generously supplied by Kenneth Peterson, LSU Medical Center, Shreveport, La.) were probed with restriction fragments generated from the strain 569B clone pGJ40 (6). Three positive clones were identified, and DNA from one of these was subcloned as an 8.0-kbp *Eco*RV fragment into the *Eco*RV site of pBluescript. The resulting plasmid, pDH8, was used as a source of DNA to produce subclones for DNA sequence analysis of the O395 *toxT* locus.

**Nucleotide sequence accession number.** The GenBank accession number for the sequences presented in this paper is L01623.

## RESULTS

**Characterization of the *V. cholerae* 569B *toxT* locus.** The plasmid pGJ40 expressing *toxT* (Fig. 1) was isolated from a library of *Sau*3A partial fragments generated from *V. cholerae* 569B and cloned into the *Bam*HI site of pBR327 (13). pGJ40 was identified by its ability to activate a *ctx-lacZ* gene fusion in *E. coli* and was subsequently shown to activate a number of other virulence-related genes from *V. cholerae* which also require ToxR for their expression, such as *tagA* (ToxR-activated gene A), *tcpA*, and *tcpC* (6). Mutational analysis of pGJ40 was done in order to identify the region of cloned *V. cholerae* DNA responsible for activation of *ctx-lacZ* (Fig. 1). This analysis showed that deletion of *Vibrio* DNA downstream of an *Xho*I site within pGJ40 abolished ToxT activity, whereas deletion of a nearby *Cla*I site did not (Fig. 1, cf. pVJ44 and pVJ61). Filling in the *Xho*I site destroyed *toxT*, while filling in the upstream *Spe*I site did not. Thus, *toxT* resides between the *Spe*I site and the first *Cla*I site downstream of it. Consistent with this, analysis of 50 Tn*phoA* insertions in pGJ40 that abolished ToxT activity showed that all but two of these mapped upstream of the *Mlu*I site shown in Fig. 1 (data not shown), indicating that *toxT* is primarily upstream of the *Mlu*I site and probably ends just downstream of it.

Expression of *toxT* cloned in pGJ40 was proposed earlier to initiate from the *tet* gene promoter of the cloning vector pBR327 (6). This was supported by the observation that insertion of Tn*phoA* into vector restriction fragments containing the *tet* gene promoter abolished ToxT activity (data not shown). Thus, these data indicate that *toxT* in pGJ40 is expressed from the *tet* promoter and extends just between the *Xho*I and *Cla*I sites shown in Fig. 1.

***toxT* encodes an AraC-like protein and is part of the *tcp* gene cluster.** The nucleotide sequence of approximately 2.5 kbp of DNA beginning just upstream of the *Bam*HI site at which *V. cholerae* DNA is inserted in pGJ40 and ending past the *Cla*I site (Fig. 1) was determined. Analysis of this DNA sequence (Fig. 2) revealed two complete open reading frames (ORFs) and the start of a third on the same strand of DNA as the *tet* gene promoter. The downstream complete ORF spans the *Xho*I site and is predicted to encode a protein 32 kDa in size. This ORF was designated *toxT*, and comparison of this ORF with the National Biomedical Research Foundation and SwissProtein data bases showed it to be similar to several transcriptional regulators from different bacteria which collectively have been designated AraC-like proteins because of

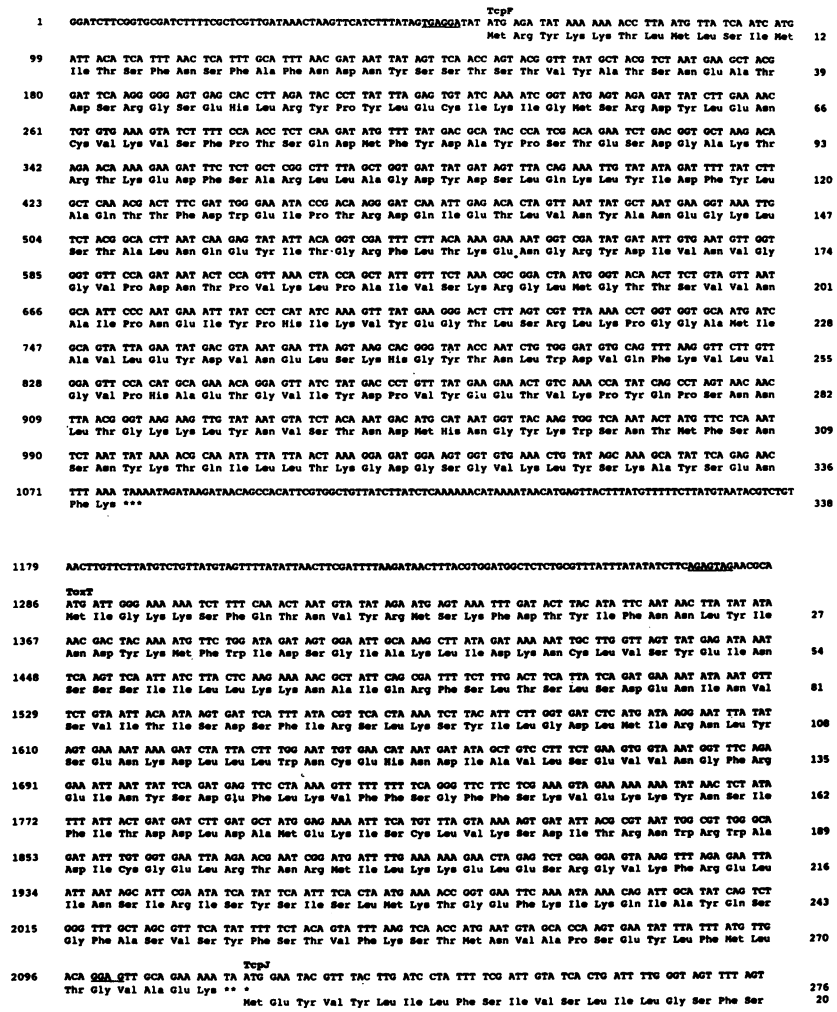

FIG. 2. Nucleotide sequences of *tcpF* and *toxT*. Nucleotide 1 is the first base of the *Sau*3A-*Bam*HI fused restriction site between *V. cholerae* and pBR327 DNAs. The ORFs representing *tcpF*, *toxT*, and the first several residues of *tcpJ* are shown. The sequence presented is for *V. cholerae* 569B. The final nucleotides of the *tcpE* coding sequence are left untranslated in the beginning part of the sequence. Putative Shine-Dalgarno ribosome binding sites are underlined.

their similarity to the arabinose operon regulator from *E. coli* and *Salmonella typhimurium* (Fig. 3) (2, 3). The greatest similarities in this family were with FapR, a regulator of the 987P pilus operon from *E. coli* (9) and VirF, a regulator controlling plasmid-encoded virulence genes in the yersiniae (2). ToxT is 19.5% identical over a 256-amino-acid overlap with FapR and is 19.2% identical over a 224-amino-acid overlap with VirF. AraC-like proteins are distinguished primarily by a conserved domain at their C termini which harbors a helix-turn-helix motif typical of DNA-binding proteins (boxed in Fig. 3) (24). Their N termini are nonsimilar and may play a role in specific interactions with effector molecules (20).

Analysis of the other ORFs encoded within this region demonstrated that *toxT* resides 210 nucleotides downstream of the *tcpF* gene and its stop codon overlaps the start codon for the *tcpJ* gene. These genes are required for biogenesis of the TCP. TcpJ is a peptidase responsible for processing the TcpA structural subunit (8), and the function of TcpF is not yet characterized, but TcpF is encoded directly downstream of the gene encoding TcpE; the DNA encoding the last 16 codons for *tcpE* are shown untranslated upstream of *tcpF* in Fig. 2. TcpE shows similarity to secretion determinants from different genera of bacteria (22).

The less stringent environmental modulation of virulence gene expression observed in strain 569B compared with that of more-typical strains was postulated to be due to differences within the *toxT* gene or its expression (4). *toxT*-containing clones were therefore isolated from a λ library of *V. cholerae* O395. DNA sequence analysis of one of these clones showed that the sequences of *tcpF* and *toxT* and their intergenic region were the same as for strain 569B. Therefore, if the phenotypic differences between 569B and O395 are the result of an alteration in *toxT* expression, the relevant genetic change resides elsewhere. To date, we have no data that would explain these differences.

**Northern blot analysis.** Previous work suggested that *toxT* expression is normally ToxR dependent. This conclusion was drawn from the observation that expression of the insert cloned in pGJ40 from the *tet* promoter was sufficient to restore *tcpA* and *ctxAB* expression to a *toxR* mutant (6). Several lines of evidence point to *toxT* and not *tcpF* or *tcpJ*

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            1                                                  50
ToxT        ...MIGKKSF QTNVYRMSKF DTYIFNNLYI NDYKMF.... ....WIDSGI
FapR        .......... ....MKLKNI HLYNYVVIYT KNCEIYINKG NEQVYIPPRM
AraC/Ecoli  .......... MAEAQNDPLL PGYSFNAHLV AGLTPIEANG YLDFFIDRPL
AraC/Salty  .......... MAETQNDPLL PGYSFNAHLV AGLTPIEANG YLDFFIDRPL
VirF        .......... .........M ASLEIIKLEW ATPIFKVVEH
CelD        MMQPVINAPE IATAREQQLF NGKNFHVFIY NKTESISGLH QHDYYE....

            51                                                 100
ToxT        AK.......L IDKNCLVSYE .....INSSS IILLKKNAIQ RFSLTSLSDE
FapR        VA.......I FEKNISFNIE T....IRKGD GVLYESFDMK HELLTSLRRV
AraC/Ecoli  GMKGYILNLT IRGQGVVKNQ GREFVCRPGD ILLFPPGEIH HYGRHPEARE
AraC/Salty  GMKGYILNLT IRGEGVINNN GEQFVCRPGD ILLFPPGEIH HYGRHPDASE
VirF        SQDG..LYIL LQGQISWQNS SQTYDLDEGN MLFLRRGS.. .YAVRCGTKE
CelD        ......FTLV LTGRYFQEIN GKRVLLERGD FVFIPLGSHH QSFYEFGATR

            101                                                150
ToxT        NINVSVITIS DSFI..R..S LKSYILGDLM IRNLYSENKD LLLWNCEHND
FapR        .IEPSVKFAA ESYTNKR..S FK........ .......EKR IFVKSC....
AraC/Ecoli  WYHQWVYFRP RAYWHEWLNW PS.IFANTGF FRPDEAHQPH F.....SDLF
AraC/Salty  WYHQWVYFRP RAYWQEWLTW PT.IFAQTGF FRPDEARQPH F.....SELF
VirF        PCQLLWIPLP GSFLSTFLHR FG.SLLSE.. IRRDNATPKP LLIFNISPIL
CelD        ILN..VGISK RFFEQHYLPL LPYCFVASQV YRTNNA.... ..........

            151                                                200
ToxT        IAVLSEVVNG FREINYSDEF L.KVF.FSGF FSKVEKK... YNSIFITDDL
FapR        .SIVIDLFKR LKD.NGSPEF T.AIYELAFL VSKCENPSMF AISLFSSVAV
AraC/Ecoli  GQII...NAG QGEGRYSELL AINLLEQLLL ....RRMEAI NESLHPPMDN
AraC/Salty  GQII...SAG QGEGRYSELL AINLLEQLLL ....RRMAVI NESLHPPMDS
VirF        SQSIQNLCAI LERSDFPSVL TQLRIEELLL LLAFSSQGAL FLSALRHLGN
CelD        ..FLTYVETV ISSLNFRETG LEEFVEMVTF YVINRLRHYR EEQVIDDVPQ

            201                                                250
ToxT        DAMEKISCLV KSDITRNWRW ADICGELRTN RMILKKELES R.GVKFRELI
FapR        TFSERIVTLL FSDLTRKWKL SDIAEEMHIS EISVRKRLEQ E.CLNFNQLI
AraC/Ecoli  RVREACQYIS DHLADSNFDI ASVAQHVCLS PSRLSHLFRQ QLGISVLSWR
AraC/Salty  RVRDACQYIS DHLADSHFDI ASVAQHVCLS PSRLSHLFRQ QLGISVLSWR
VirF        RPEERLQKFM EENYLQGWKL SKFAREFGMG LTTFKELFGT VYGISPRAWI
CelD        WLKSTVEKMH DKEQFSESAL ENMVALSAKS QEYLTRATQR YYGKTPMQII

            251                                                300
ToxT        NSIRISYSIS LMKTGEFK[IK QIAYQSGFAS VSYFSTVFKS TMNVAP]SEYL
FapR        LDVRMNQAAK FIIRSDHQ[IG MIASLVGYTS VSYFIKTFKE YYGVTP]KKFE
AraC/Ecoli  EDQRISQAKL LLSTTRMP[IA TVGRNVGFDD QLYFSRVFKK CTGASP]SEFR
AraC/Salty  EDQRISQAKL LLSTTRMP[IA TVGRNVGFDD QLYFSRVFKK CTGASP]SEFR
VirF        SERRILYAHQ LLLNGKMS[IV DIAMEAGFSS QSYFTQSYRR RFGCTP]SQAR
CelD        NEIRINFAKK QLEMTNYS[VT DIAFEAGYSS PSLFIKTFKK LTSFTP]KSYR

            301          315
ToxT        FMLTGVAEK. .....
FapR        I...GIKENL RCNR.
AraC/Ecoli  AGCEEKVNDV AVKLS
AraC/Salty  AGCE..... .....
VirF        LTKIATTG.. .....
CelD        KKLTEFNQ.. .....

                        'Helix-Turn-Helix' Motif Consensus:
[L,I,V]-x2-[L,I,V]-x4-[G]-[I,F,Y]-x5-[F]-x3-[F,Y]-x7-[P]
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FIG. 3. Alignment of ToxT and other AraC family members. AraC-like proteins from other bacterial species were aligned by using the PILEUP program in the University of Wisconsin Genetics Computer Group (GCG) package. The helix-turn-helix sequence was generated by using the MOTIFS program in the GCG package. Sequences shown represent AraC proteins from *E. coli* and *S. typhimurium* (2), VirF from *Yersinia enterocolitica* (3), FapR from *E. coli* (9), and CelD (17), a negative regulator of the *cel* operon in *E. coli*.

as being responsible for this phenotype. First, the two *tcp* gene products are predicted to be periplasmically located (8, 22) with no detectable similarity to other transcriptional activators (9), while ToxT shows strong similarity to a family of prokaryotic regulatory gene products. Second, Tn*phoA* insertions within *tcpF* in *V. cholerae* do not abolish TcpA production (22). If *tcpF* were a component of ToxT activity, such mutations would be expected to abolish TcpA synthesis. Third, a frameshift mutation in *toxT* completely abolishes ToxT activity, whereas a frameshift mutation within the *tcpF* gene does not.

The pattern of *toxT* expression in *V. cholerae* was determined by Northern blot analysis. RNA was isolated from overnight cultures of wild-type or *toxR* mutant *V. cholerae* grown in medium with a pH of 6.5 at 30°C, which favors expression of ToxR-regulated genes (14). In wild-type strain O395, a transcript of approximately 1,400 nucleotides was detected with the *tcpEF* probe, whereas no detectable message was detected in the *toxR* mutant JJM43 with the same probe (Fig. 4). When a *toxT* probe of equivalent specific activity was used to analyze the same batch of RNA, a diffuse and relatively weaker signal beginning at a slightly smaller size than that seen with the *tcpEF* probe was observed in O395 RNA, and only after the *tcpEF* specific signal had been overexposed (Fig. 4). As with *tcpEF*, no RNA was detected with the *toxT* probe in the *toxR* mutant strain (Fig. 4). When wild-type cells were grown at pH 8.5, a condition that down-regulates ToxR-dependent gene expression, no signal was detected with either probe (data not shown). The patterns of hybridization demonstrated in Fig. 4 were consistently observed when other probes encompassing this region were used and when different batches of RNA were analyzed.

## DISCUSSION

Previous work on the ToxR system of virulence gene expression in *V. cholerae* led to a model in which hierarchical expression of activators controls a number of genes (4–6). Data presented in this report support this model by

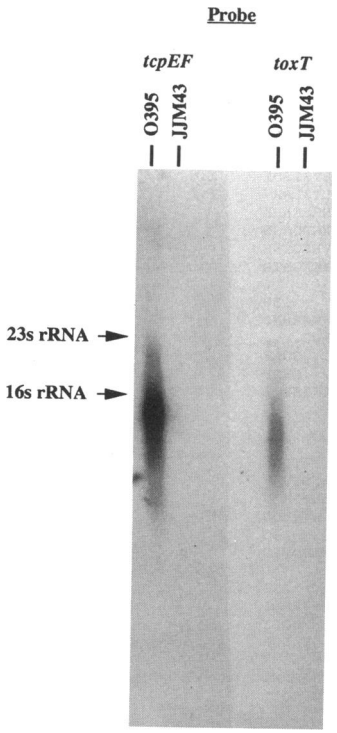


FIG. 4. Northern blot analysis of *V. cholerae*. RNA was isolated after overnight growth at 30°C in Luria-Bertani medium with a starting pH of 6.5, and Northern analysis was done as described in Materials and Methods. Restriction fragments corresponding to *tcpEF* or *toxT* are indicated. Strain JJM43 is a *toxR* null mutant. The location of the 23 and 16S rRNA subunits are shown and were identified by ethidium staining lanes with equal amounts of RNA loaded. These RNAs migrate in these gels to the same location as do rRNAs from *E. coli*, which are 2,904 and 1,541 nucleotides long, respectively.

demonstrating that ToxR, itself a member of the OmpR family of activators, is required for expression of *toxT*, which is demonstrated here to be similar to AraC-like activators identified in other genera of bacteria. AraC-like activators are similar to one another in having a C-terminal helix-turn-helix domain typical of DNA-binding proteins, but N termini within the family are dissimilar. This latter domain may function as a site of effector interaction (20), so perhaps ToxT is a sensor of some intracellular signal generated under conditions in which the ToxR regulon is to be expressed.

The finding that *toxT* resides within the cluster of genes encoding the biogenesis functions of the TCP is reminiscent of the structure of the 987P pilus gene cluster in *E. coli*, in which the gene encoding the AraC-like regulator FapR is located adjacent to the pilus cluster (9). This finding also adds a layer of complexity to understanding how coordinate control of virulence genes works in *V. cholerae*. While the location of *toxT* within the *tcp* cluster may suggest that the *toxT* product might have evolved as a regulator specific to TCP production, ToxT can activate other ToxR-regulated genes not involved in TCP biogenesis (6) and is thus a global regulator, like VirF in yersiniae (3).

Regulated expression of *toxT* is apparently an important step in the signal transduction pathway controlled by ToxR (6). This conclusion is based on the observations that introduction of *toxT* under constitutive control of the *tet* promoter into a ToxR mutant of *V. cholerae* restored expression of both TcpA and CtxB and that this expression was independent of pH, which modulates ToxR-regulated gene expression in wild-type cells (6). Northern blots in that study were done with the large *Eco*RV fragment of pGJ40 beginning in pBR327 DNA and proceeding past *toxT* (and *tcpJ*; Fig. 1) or an *Eco*RI fragment beginning in pBR327 and ending within the *toxT* coding sequence (73 nucleotides downstream of the *Xho*I site in Fig. 1) used as a probe. The abundant transcript identified in that study was probably the same one identified here with the *tcpEF* probe, but, while less abundant, *toxT* mRNA is subject to the same ToxR-dependent pH modulation. Thus ToxR is responsible for pH-sensitive expression of *toxT*.

How this control is accomplished is not clear. ToxR activation of *ctxAB* transcription requires the ability of ToxR to bind to a tandemly repeated heptamer, TTTTGAT (15), but this sequence is not present upstream of *toxT* (Fig. 2), nor is it found within 110 nucleotides upstream of *tcpE*, which is directly upstream of *tcpF* (22). A sequence within the intergenic region between *tcpF* and *toxT* is identified by computer analysis as a putative prokaryotic promoter element, but whether this actually functions as a promoter is not certain. Since insertions of Tn*phoA* into the *tet* promoter of pGJ40 abolish *toxT* activity, it follows that *tcpF* and *toxT* are (or at least can be) cotranscribed, although it has not been ruled out that *toxT* is controlled by its own promoter. Given that the stop codon for *tcpE* is only 9 nucleotides upstream of the start codon for *tcpF*, these two genes may be transcribed as a polycistronic mRNA (22). Similarly, *toxT* and *tcpJ* overlap one another's coding sequence (Fig. 2), suggesting that they may be cotranscribed. A polycistronic *tcpEF* transcript would have a predicted size of approximately 2,100 nucleotides, and a polycistronic *toxT-tcpJ* transcript would have a size of approximately 1,650 nucleotides. The sizes of the mRNAs we observed in this study are inconsistent with these predicted sizes, but since the analysis presented here was done with mRNA isolated from overnight cultures, we cannot rule out the possibility that there is processing of larger ToxR-regulated transcripts within this region.

The DNA between *tcpF* and *toxT* contains extensive inverted repeats (Fig. 5A) which would form an unusual structure with a high negative free energy (Fig. 5B). The function of this structure or whether it forms at all is unknown. While it bears certain features typical of type I transcriptional terminators (7), it is different from them in other, perhaps critical, ways. As is often seen in type I terminators, one of the predicted stem-loops ends in a run of thymine residues; however, this stem-loop is A-T rich rather than G-C rich, giving it an overall low free energy. Whether this structure acts as a transcription terminator or plays some other role in RNA metabolism, as an RNase processing site, for instance, is under investigation.

We envision three explanations, which are not exclusive of one another, that may account for some of the observations regarding expression of *tcpF* (and probably *tcpEF*) and *toxT* presented here. First, the *toxT* expression from pGJ40 that we observe may simply be the result of having *tcpF-toxT* cloned downstream of a strong, constitutive promoter, and perhaps *toxT* has its own promoter as well. Second, ToxR-regulated transcription may begin somewhere upstream of *tcpF* and terminate predominantly after *tcpF* prior to *toxT* (perhaps as a function of the stem-loop structure discussed above), but a low percentage of transcripts might

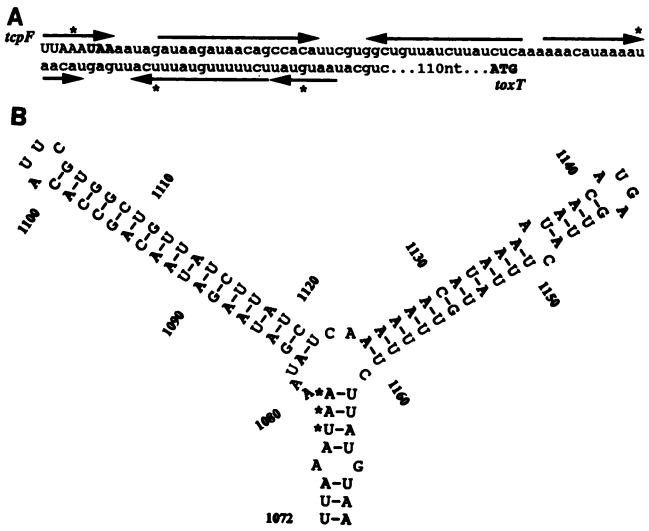


FIG. 5. Nucleotide sequence of the *tcpF-toxT* intergenic region. (A) Sequence and locations of inverted repeat sequences between the *tcpF* and *toxT* genes. Codons for *tcpF* and *toxT* are shown in uppercase letters. Asterisks represent bases that are unpaired in the corresponding stem-loop. (B) Predicted stem-loop structure (length, 98 bp; energy, −33.7 kcal/mol) formed from the sequence depicted in A, generated by the FOLD program in the University of Wisconsin GCG package. Numbering of nucleotides is as in Fig. 2. Asterisks indicate stop codon of *tcpF*.

read through to *toxT*. The absolute amount of *toxT* expression would then increase as the expression of the upstream promoter was elevated. This sort of read-through control of a transcriptional regulator has been proposed to control expression of the *kdpDE* regulatory genes from *E. coli* (19) and the *prfA* gene from the gram-positive pathogen *Listeria monocytogenes* (10). Although there are analogies with the *toxT* system to be drawn, the RNA species identified by both *tcpEF* and *toxT* probes are not the size expected for a read-through transcript encoding them both. Third, portions of a larger ToxR-regulated precursor transcript may exhibit differential stabilities. A stem-loop between *lacZ* and *lacY* in *E. coli* has been proposed to protect the *lacZ* mRNA from 3′ exonucleolytic degradation that removes the downstream portion of the *lacZYA* transcript (16). Differential stability of portions of a polycistronic mRNA has also been demonstrated for the *pap* pilus genes in *E. coli*, in which mRNA for a regulatory gene, *papB*, is encoded on the same transcript as the *papA* structural gene but is maintained in a lower concentration by rapid decay (1). In this system, a stem-loop structure downstream of *papA* has been proposed to act as a barrier to decay of the *papA* transcript (1).

The central issue that arises from all of these possibilities is the precise role played by ToxR, which has not been demonstrated to activate any of the *tcp* genes. Thus, its requirement for regulated expression of *toxT* in the activator cascade model remains unexplained. Experiments are under way to investigate the various possibilities presented above, and results from them will undoubtedly help elucidate the role of ToxR in *toxT* expression.

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## ADDENDUM IN PROOF

The *toxT* gene is the same as the *tcpN* gene, whose sequence was recently presented by Ogierman and Manning (Gene **116**:93–97, 1992).

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