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Analysis of an Autoregulatory Loop Controlling ToxT, Cholera Toxin, and Toxin-Coregulated Pilus Production in *Vibrio cholerae*

ROSA R. YU[1] AND VICTOR J. DiRITA[1,2]*

Department of Microbiology and Immunology[1] and Unit for Laboratory Animal Medicine,[2] University of Michigan Medical School, Ann Arbor, Michigan 48109

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Coordinate expression of many virulence genes in the human pathogen *Vibrio cholerae* is controlled by the ToxR, TcpP, and ToxT proteins. These proteins function in a regulatory cascade in which ToxR and TcpP, two inner membrane proteins, are required to activate *toxT* and ToxT is the direct activator of virulence gene expression. ToxT-activated genes include those whose products are required for the biogenesis of cholera toxin (CTX) and the toxin-coregulated pilus, the major subunit of which is TcpA. This work examined control of *toxT* transcription. We tested a model whereby activation of *toxT* by ToxR and TcpP is required to prime an autoregulatory loop in which ToxT-dependent transcription of the *tcpA* promoter reads through a proposed terminator between the *tcpF* and *toxT* genes to result in continued ToxT production. Primer extension analysis of RNA from wild-type classical strain O395 showed that there are two products encoding *toxT*, one of which is longer than the other by 105 bp. Deletion of the *toxT* promoter ($toxT_{\Delta pro}$) resulted in the abolishment of *toxT* transcription, as predicted. Deletion of the *tcpA* promoter ($tcpA_{\Delta pro}$) had no effect on subsequent detection of the smaller *toxT* primer extension product, but the larger *toxT* product was not detected, indicating that this product may be the result of transcription from the *tcpA* promoter and not of initiation directly upstream of *toxT*. Neither mutant strain produced detectable TcpA, but the CTX levels of the strains were different. The $toxT_{\Delta pro}$ strain produced little detectable CTX, while the $tcpA_{\Delta pro}$ strain produced CTX levels intermediate between those of the wild-type and $toxT_{\Delta pro}$ strains. Dependence of *toxT* transcription on TcpP and TcpH was confirmed by analyzing RNAs from strains carrying deletions in the genes encoding these regulators. The *tcpP* defect resulted in undetectable *toxT* transcription, whereas the *tcpH* mutation led to a diminishing of *toxT* RNA but not complete abolishment. Taken together, these results suggest that *toxT* transcription is dependent on two different promoters; one is directly upstream and is activated in part by TcpP and TcpH, and the other is much further upstream and is activated by ToxT.

Vibrio cholerae is the causative agent of the diarrheal disease cholera, which is usually acquired by oral ingestion of the bacterium with contaminated water or food (10). In response to specific environmental conditions, such as temperature, pH, or osmolarity (11, 23), *V. cholerae* expresses several virulence determinants, including the cholera toxin (CTX), a toxin-coregulated pilus (TCP), the accessory colonization factor, and a major outer membrane protein (OmpU) (24, 28, 34, 36). CTX is the best-characterized virulence factor and is composed of a single A subunit and five identical B subunits (12, 27). The enzymatically active A subunit is predominantly responsible for fluid loss through an ADP-ribosylation mechanism that results in constitutive cyclic-AMP (cAMP) production in host cells, leading to the opening of normally gated channels (1). Environmental signals optimal for CTX production also stimulate the expression of TcpA (17, 19) and OmpU, a porin that may also function as an adhesin (4, 33, 34). TCP is a pilus in the type IV family that is essential for colonization and virulence. It is made up of a single protein encoded by the *tcpA* gene, which is part of a pathogenicity island that includes other *tcp* genes whose products are involved in the biogenesis of the pilus structure, as well as the *acf* genes (18, 19, 26, 28).

Coordinate expression of *ctxAB*, *tcpA* to *F*, and some *acf* genes is due to the action of several regulatory proteins. In the current model, these proteins function in a branched regulatory cascade in which two activator proteins, ToxR and TcpP, are required for activation of *toxT* transcription (13, 32) and ToxT, a member of the AraC family of transcriptional activators (15), activates the expression of other virulence genes, including *ctxAB* and *tcpA* to *F* (9, 32). The *ompU* gene is in a ToxT-independent branch of this cascade and is activated directly by ToxR (5, 6).

Both ToxR and TcpP are inner membrane proteins with cytoplasmic DNA-binding domains homologous to members of the two-component family of transcriptional activators found in various species of bacteria (13, 24, 25). Each of these proteins is encoded by an operon by which another membrane protein is encoded. For ToxR, this protein is ToxS, and for TcpP, it is TcpH (3, 8, 21). ToxS and TcpH act as effector proteins for ToxR and TcpH, respectively, through a mechanism that likely involves periplasmic interaction between the regulator and the effector (3, 8). The precise mechanism by which ToxRS and TcpPH control *toxT* transcription is not understood. One observation that may eventually contribute to a better understanding of this system is that overexpression of TcpP suppresses a *toxR* mutant for *toxT* expression, while overexpression of ToxR does not suppress a *tcpP* mutant (13, 14, 19a).

This work examined the transcription of *toxT* in the context of its location at the end of the *tcp* gene cluster. Previous genetic analysis demonstrated that insertion mutations in the

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109. Phone: (734) 936-3804. Fax: (734) 936-3235. E-mail: vdirita@umich.edu.

tcpA gene resulted in downregulation of the production of both TCP and CTX (2). This was interpreted as being due to the polar effects of the insertions on *toxT*, because RNase protection experiments showed that the *tcp* gene cluster, including *toxT*, was transcribed as a long polycistronic message (2). In addition, it was recently shown that the cAMP-cAMP receptor protein complex plays a negative role in regulation of toxin production, potentially through repression of *tcpA* transcription, which may result in decreased *toxT* transcription through transcriptional polarity (31, 32). Other work demonstrated that ToxR-dependent activation of *toxT* occurs at a promoter that is immediately upstream of *toxT* (14) and that a transcription terminator with 80% efficiency precedes the *toxT* gene downstream of *tcpF*.

In one model that would account for these observations, ToxR (in conjunction with TcpP) activates *toxT* from the proximal promoter and ToxT activation of the *tcpA* promoter contributes to subsequent expression of the gene through readthrough of the relatively inefficient transcription terminator (compared to a well-characterized λ terminator) between *tcpF* and *toxT* (14). This model makes specific predictions about the contributions of different regulatory elements within the *tcp* gene cluster, and in this work, we tested some of those predictions.

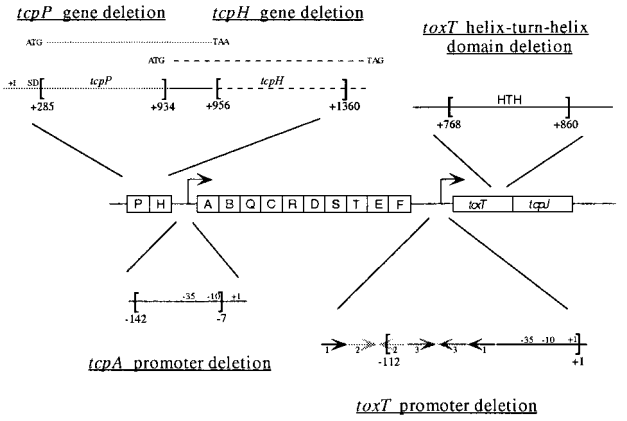


FIG. 1. Schematic diagram of the *V. cholerae tcp* gene cluster. Shown in the center is the organization of the *tcp* gene cluster of wild-type *V. cholerae*. Each box represents a gene. The right-angle arrows represent promoters. Certain regions are enlarged to show the details of each deletion. Brackets indicate the deleted sequence, and the number below each bracket is the nucleotide position, relative to the +1 start site for transcription, which represents the start or the end of a deletion. Arrows at the *toxT* promoter region denote three pairs of inverted repeats. *tcpP* and *tcpH* are in an operon, and their ORFs overlap. The ORFs are shown above the operon. The leftward-pointing arrows beneath *toxT* and *tcpA* represent primers used in the RNA primer extension analyses. SD, Shine-Dalgarno region; HTH, helix-turn-helix motif.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. cholerae* and *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 30 or 37°C. Strains were maintained at −70°C in LB medium with 20% glycerol. Antibiotics were used at the following concentrations (unless otherwise stated): ampicillin, 100 μg/ml; streptomycin, 100 μg/ml; kanamycin, 30 μg/ml. Plasmids were introduced into *E. coli* strains by either transformation or electroporation and into *V. cholerae* strains by conjugation.

DNA manipulation. Plasmid DNA was purified with Qiagen columns (Qiagen, Inc.). Cloning was performed by using standard protocols (29). PCR was performed with the Expand High Fidelity PCR System (Boehringer Mannheim) by using the manufacturer's protocols. A recombinant PCR method, gene splicing by overlap extension (16), using primers within and surrounding the *toxT* promoter region, the *tcpA* promoter region, *tcpP*, and *tcpH*, was performed to create deletions of the corresponding regions (for a description of the construction of $toxT_{\Delta hth}$, see reference 5). The DNA templates (15, 36) and primers used for construction of each mutant are listed in Table 1. This procedure requires two PCR rounds, as follows (using the *toxT* promoter deletion as an example). Outside primer 1 is complementary to a sequence in *tcpF* (upstream of the *toxT* promoter), and primer 4 is complementary to a sequence in *toxT* (downstream of the *toxT* promoter), and they both have restriction endonuclease sites inserted to mediate cloning. Inside overlapping primers 2 and 3 were designed to delete the *toxT* promoter region, and they are partly complementary to the sequence upstream and partly complementary to the sequence downstream of the desired deletion. First, two PCRs were carried out, one with primers 1 and 2 and the other with primers 3 and 4. The product of each reaction was purified by agarose gel electrophoresis, followed by gel extraction with the QIAEX II gel extraction system (Qiagen, Inc.), mixed together, and used as the template for a second round of PCR with outermost primers 1 and 4. The amplified PCR fragment was cloned into the corresponding sites of positive-selection suicide plasmid pKAS32, which cannot replicate in *V. cholerae* in the absence of the Pir protein (30).

The resulting plasmid was first introduced into *E. coli* MC4100λpir or DH5αλpir by electroporation, transformed into *E. coli* SM10λpir, and finally

TABLE 1. DNA template and primers used for construction of mutants in this study[a]

Strain	DNA template (reference)	Primer 1	Primer 2	Primer 3	Primer 4
$toxT_{\Delta pro}$ mutant	pDH8 (15)	5′-CCGGAATTCGAAAATGGTCGATATGAT-3′ (*Eco*RI)	5′-AGTTATCTTAAAATCGCGAATGTGGCTGTTA-3′	5′-TAACAGCCACATTCGCGATTTTAAGATAACT-3′	5′-CCGGAATTCTACTTTCGAGAAGAACCC-3′ (*Eco*RI)
$tcpA_{\Delta pro}$ mutant	ptcpA::phoA2-1 (36)	5′-GCTCTAGACAGACAGATCCACAAGGT-3′ (*Xba*I)	5′-AGCAACACGCACGGTACCGGCCAACTTATTCAATTC-3′ (*Kpn*I)	5′-AATAAGTTGGCCGGTACCGTGCGTGTTGCTTACGTT-3′ (*Kpn*I)	5′-TCCCCGCGGTGCAATATATGGGAACAT-3′ (*Sac*II)
Δ*tcpP* mutant	O395 chromosomal DNA	5′-GGGGTACCGATAACTTTGCAACCGTT-3′ (*Kpn*I)	5′-TAATTTTTTGTGCATTACTTTACATTTTCT-3′	5′-AGAAAATGTAAAGTAATGCACAAAAAATTA-3′	5′-TCCCCGCGGGACGATCTCAATACAACT-3′ (*Sac*II)
Δ*tcpH* mutant	O395 chromosomal DNA	5′-GGGGTACCATAAAAAAATGGGTCGTT-3′ (*Kpn*I)	5′-ACACTATCTAGGCGGAGCTTTTAATTTTTT-3′	5′-AAAAAATTAAAAGCTCCGCCTAGATAGTGT-3′	5′-TCCCCGCGGTGTTCTTCTTTTACAAAT-3′ (*Sac*II)

[a] Restriction sites for each individual primer are underlined and in parentheses if applicable. All primers were designed by using the sequences under GenBank accession no. X64098.

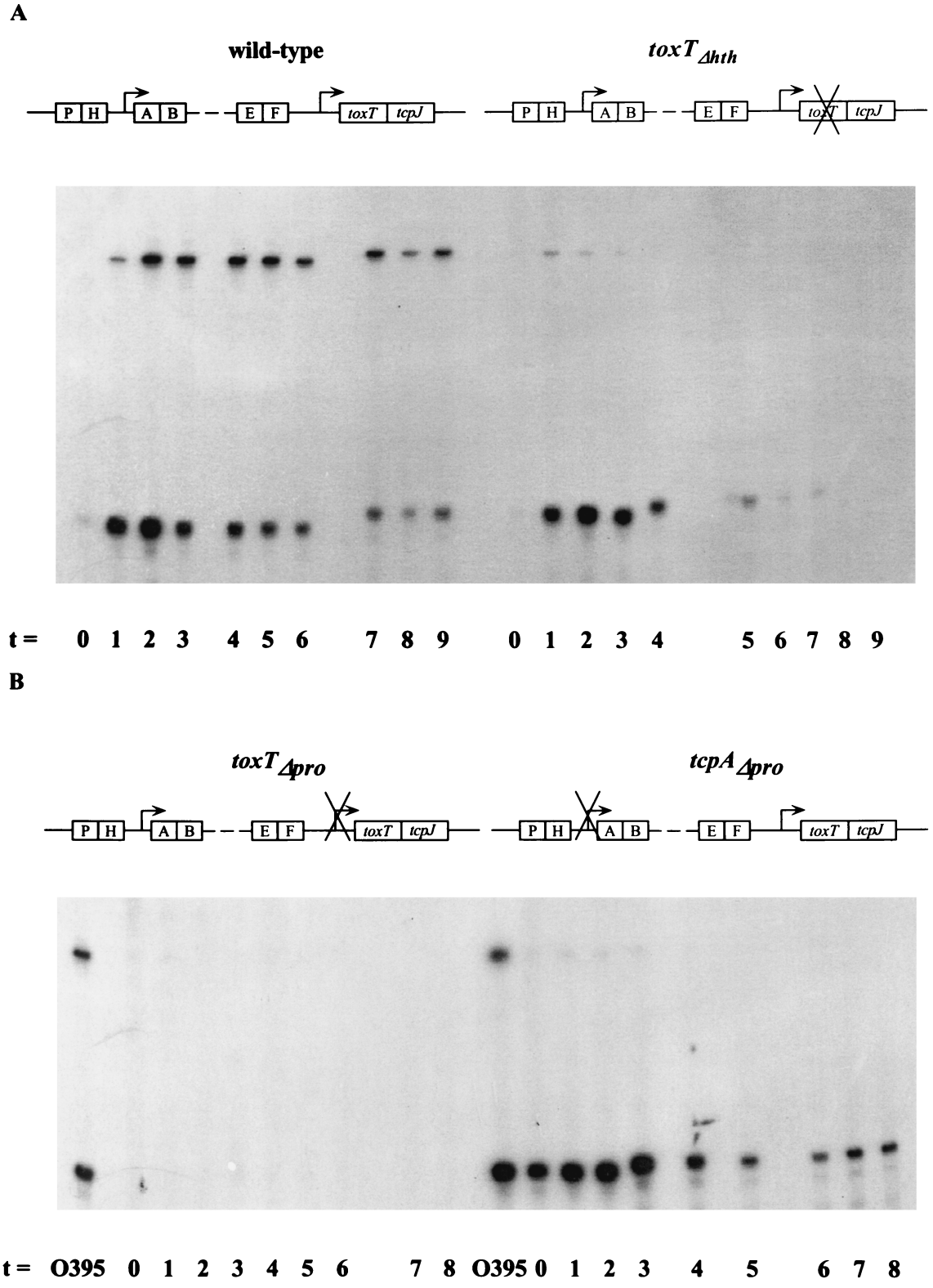


FIG. 2. RNA primer extension analyses of *V. cholerae* wild-type O395 and a $toxT_{\Delta hth}$ mutant strain (A) and $toxT_{\Delta pro}$ and $tcpA_{\Delta pro}$ mutant strains (B). Samples were collected at 1-h intervals after back dilution of an overnight culture grown in LB medium at 30°C 1:100 into fresh LB medium. A radiolabeled *toxT* primer was used. RNA collected from wild-type strain O395 after 2 to 3 h of growth was used as a positive control for the mutant strains in panel B.

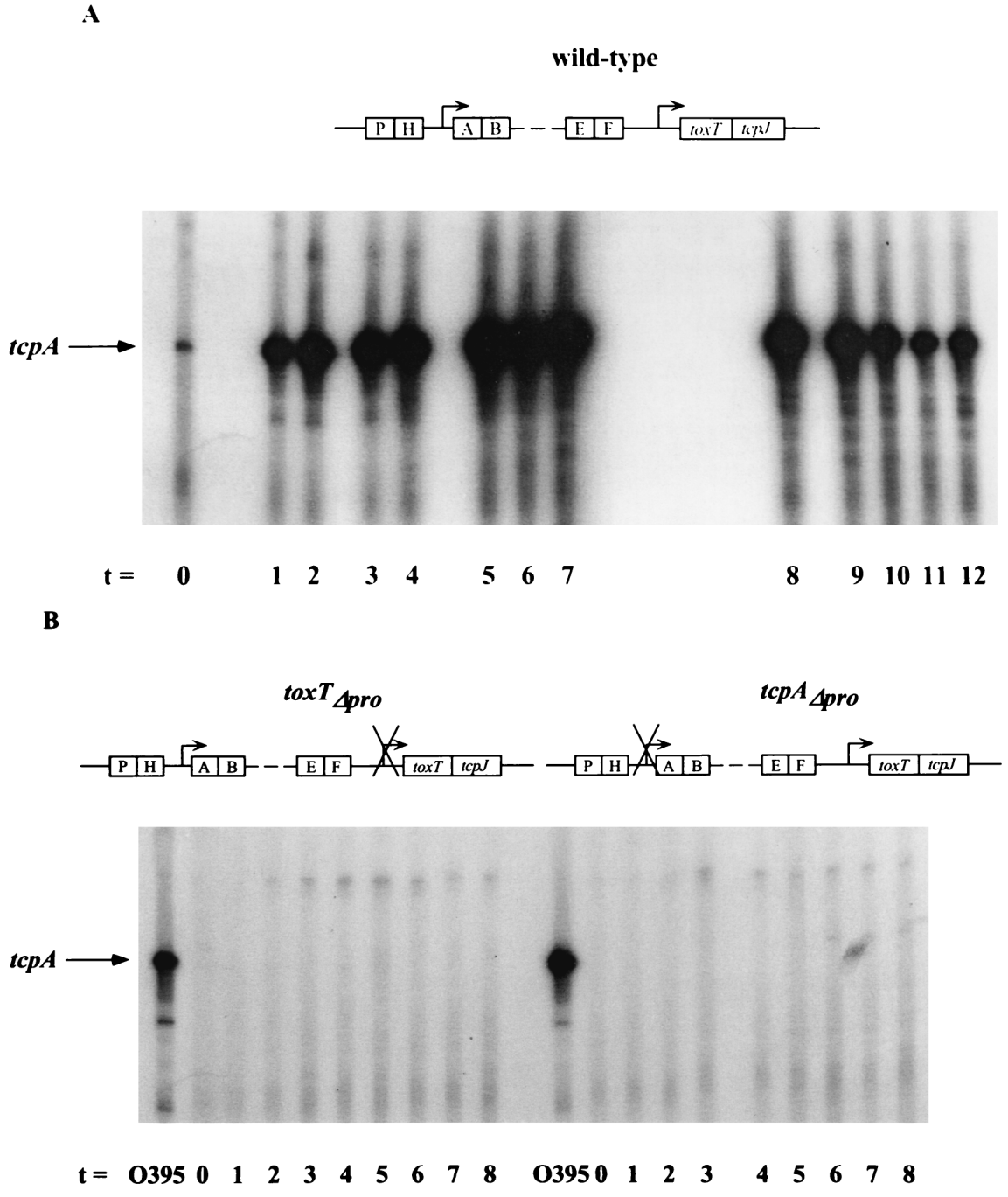
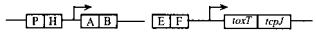
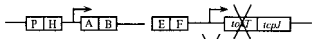
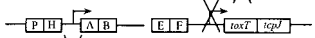
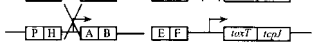
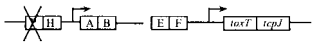
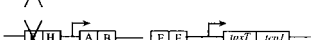
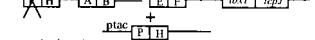


FIG. 3. RNA primer extension analyses of *V. cholerae* strain O395 wild-type (A) and mutant strains $toxT_{\Delta pro}$ and $tcpA_{\Delta pro}$ (B). Samples were collected at 1-h intervals after back dilution of an overnight culture grown in LB medium at 30°C 1:100 into fresh LB medium. A radiolabeled *tcpA* primer was used. RNA collected from wild-type strain O395 after 2 to 3 h of growth was used as a positive control for the mutant strains in panel B. The position of the *tcpA* transcript is indicated.

introduced into *V. cholerae* classical strain O395 by conjugation from SM10λpir. The transconjugants were selected on TCBS agar (Difco) for plasmid-encoded ampicillin (50 μg/ml) resistance. Single recombinants that have the plasmid integrated into the chromosome were grown in the absence of antibiotic selection and then selected again on LB agar containing streptomycin (1 mg/ml) for strains that have undergone resolution of the cointegrate by recombination (30). DNAs from isolates that were both sensitive to ampicillin and resistant to streptomycin were analyzed by PCR using outermost primers 1 and 4. DNA amplified with these primers gave a smaller fragment from DNA of the deletion mutant than from that of the wild type.

RNA analysis. For primer extension analysis, an overnight culture of *V. cholerae* grown in LB medium at 30°C to late log phase (optical density at 600 nm [OD_{600}], >3.0) was subcultured 1:100 into fresh LB medium. After back dilution ($t = 0$) and at 1-h intervals, aliquots of the cultures were removed and poured over crushed ice prior to centrifugation for cell recovery. Cell pellets were stored at −20°C until ready for RNA isolation. RNA was obtained from cultures of *V. cholerae* by using TRIzol Reagent (Gibco BRL) by following the manufacturer's protocols. The RNA samples were then treated with DNase I and quantified by determination of the OD_{260}. The same amount of RNA was used for primer extension as previously described (14), by using a *toxT*-specific primer (5′-CAT

TABLE 2. Toxin production in *V. cholerae* wild-type and mutant strains[a]

Strain	Relevant genetic map	Avg toxin production (ng/ml/OD_{600}) ± SD[b]
O395 (wild type)		1,370 ± 130
toxT$_{\Delta hth}$ mutant		0.038 ± 0.015
toxT$_{\Delta pro}$ mutant		0.027 ± 0.0021
tcpA$_{\Delta pro}$ mutant		760 ± 150
Δ*tcpP*/pMMB66EH mutant[c]		0.043 ± 0.031
Δ*tcpP*/p*tcpPH* mutant[c]		5,670 ± 720
Δ*tcpH* mutant		19 ± 3

[a] Strains were grown overnight in LB medium, and supernatants were assayed by GM_1-ganglioside ELISA.
[b] Toxin levels represent three individual experiments.
[c] Plasmid experiments included 1 mM IPTG for induction of genes under *tac* promoter control.

TAGTTTGAAAAGATTTTTTCCCAATCAT-3′) or a *tcpA*-specific primer (5′-TTCTTTTACAAATTTCTTCTTAAAAAGCTGTTTTAA-3′).

Protein analysis. Total cell lysates were prepared from *V. cholerae* cells grown to stationary phase overnight (with 1 mM isopropyl-β-D-thiogalactopyranoside [IPTG] if necessary). Samples (1 ml) were removed from the cultures and centrifuged. The harvested pellet was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, normalized for OD_{600}, and boiled for 5 min. Aliquots of this lysate were subjected to electrophoresis on a 12% polyacrylamide gel with a 5% stacking gel. For Western blotting, the gels were blotted onto nitrocellulose and probed with anti-TcpA peptide 6 rabbit polyclonal antibodies kindly supplied by Ron Taylor (Dartmouth Medical School).

CTX levels were quantified from supernatants of cultures by the GM_1-ganglioside enzyme-linked immunosorbent assay (ELISA) (35) using anti-CtxB rabbit polyclonal antibodies kindly supplied by Michael Bagdasarian (Michigan State University).

RESULTS

Transcription of *toxT* in the *tcp* gene cluster. To analyze transcription of *toxT*, we used derivatives of classical strain O395 carrying deletions of key elements in the *tcp* gene cluster (Fig. 1). We had observed that wild-type strain O395 produces little detectable *toxT* mRNA after overnight growth and exploited this observation to establish a time course experiment as shown in Fig. 2A. When primer extension of *toxT* RNA was done on samples prepared from wild-type strain O395 after 1:100 back dilution of an overnight culture, we observed that both of the primer extension products previously described (14) became detectable within an hour after back dilution. The smaller primer extension product (99 bp) has been previously shown to initiate from a ToxR-dependent promoter immediately preceding the *toxT* open reading frame (ORF) (14). The source of the larger product (204 bp) is less clear, but it has been speculated to arise from either an RNA-processing event or a block to reverse transcription due to potential secondary structure in the RNA at that position (14, 15). In this experiment, the smaller product was more abundant within the first hour, but by 2 h, the two products were roughly equivalent. By 5 to 9 h after back dilution, each began to diminish in prevalence (Fig. 2A) and after 9 h, they became undetectable (data not shown).

In order to determine whether the transcripts shown in Fig. 2A require de novo transcription or accumulated over the time of the experiment, rifampin was added to the cultures 2 h after back dilution. When this was done, RNA diminished in abundance very rapidly with a half-life of less than 5 min for each, as judged by primer extension (data not shown), indicating that new transcription is continuously required to generate the pattern shown in Fig. 2A.

Analysis of *tcpA* transcription in the wild-type strain by primer extension showed that immediately after the back dilution, *tcpA* mRNA (120 bp) was minimal but increased steadily after that until 7 h, when its prevalence as a percentage of the total mRNA began to decrease (Fig. 3A). This is similar to the transient nature of *toxT* mRNA production shown in Fig. 2A.

In order to ascertain whether functional ToxT is required for the wild-type pattern of *toxT* transcription, a similar time course experiment was performed by using RNA from a derivative of strain O395 that expresses a null allele of *toxT*, called *toxT*$_{\Delta hth}$, that lacks the predicted helix-turn-helix DNA-binding domain of ToxT (5). In contrast to what we observed in the wild type, in the *toxT*$_{\Delta hth}$ strain, the larger primer extension product was severely diminished in quantity, while the smaller product was produced at levels similar to the wild-type level (Fig. 2A). Thus, transcription of *toxT* from a near promoter is not dependent on functional ToxT. This result also shows that the larger product requires a promoter that is ToxT dependent.

We next addressed specifically the consequence of ToxR-dependent activation of the *toxT* promoter. To do this, we analyzed a strain in which the previously mapped ToxR binding site in the *toxT* promoter (14) had been deleted. This strain, *toxT*$_{\Delta pro}$, was predicted not to synthesize any *toxT* from the near promoter due to the lesion in the ToxR binding site and the basal promoter element, and that is what we observed in the primer extension experiment shown in the left side of Fig. 2B. No larger primer extension product was detected in this experiment.

We observed no detectable *tcpA* mRNA in the *toxT*$_{\Delta pro}$ strain (Fig. 3B), which clearly demonstrates the cascade of regulation in the ToxR regulon: activation of *tcpA* transcription is ultimately dependent on activation of *toxT* by ToxR. That the phenotype of the *toxT*$_{\Delta pro}$ strain produces undetectable levels of toxin is worth noting (Table 2), as it strongly implies that there is no source of ToxT in the cell in the absence of ToxR-dependent transcription. Lack of toxin production by the *toxT*$_{\Delta pro}$ strain also confirms an earlier observation that functional ToxT must be produced in *V. cholerae* in order for the *ctxAB* promoter to be activated (5), irrespective of the fact that ToxR alone can activate *ctxAB* transcription when tested in *E. coli* (22).

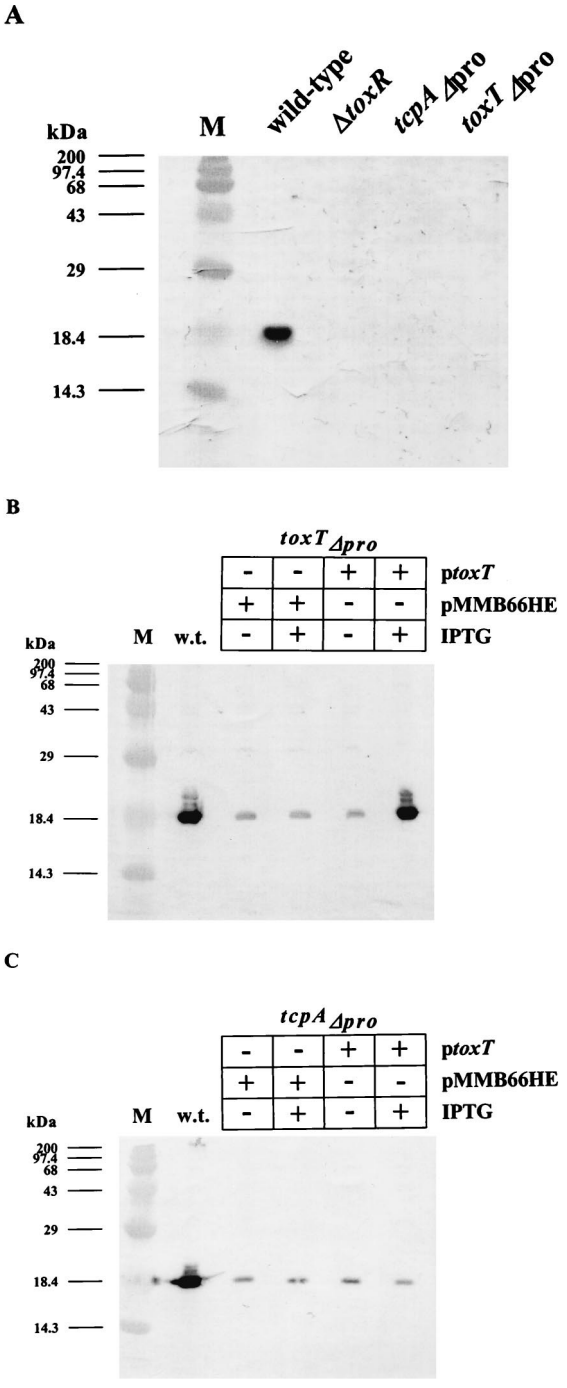


FIG. 4. Western blot analysis using anti-TcpA antibody. *V. cholerae* wild-type O395 and mutant strains Δ*toxR*, $toxT_{\Delta pro}$, and $tcpA_{\Delta pro}$ (A); wild-type O395 and mutant strain $toxT_{\Delta pro}$/pMMB66HE or $toxT_{\Delta pro}$/p*toxT* (B); and wild-type O395 and mutant strain $tcpA_{\Delta pro}$/pMMB66HE or $tcpA_{\Delta pro}$/p*toxT* (C) were grown overnight in LB medium at 30°C. IPTG (1 mM) was added to induce *toxT* transcription in strains carrying plasmids. p*toxT* harbors the *toxT* ORF on low-copy-number plasmid pMMB66HE under the control of the IPTG-inducible *tac* promoter. Protein molecular size standards (lane M) are indicated on the left. w.t., wild type.

The data presented above suggest that the larger *toxT* primer extension product arises from a ToxT-dependent transcript. The logical site for initiation of this transcript is at the *tcpA* promoter. To test this hypothesis, we performed *toxT* primer extension on RNA from a strain in which the *tcpA* promoter had been deleted ($tcpA_{\Delta pro}$). The smaller, ToxR-dependent product was observed (Fig. 2B), whereas the larger product was nearly undetectable. To confirm that the deletion had, in fact, abolished *tcpA* transcription, we performed primer extension on the same RNA samples by using a *tcpA*-specific primer and observed no *tcpA* primer extension product (Fig. 3B).

Figure 4 shows TcpA immunoblot data confirming the phenotypes of the mutant strains described above. The blot in panel A demonstrates the dependence of TcpA production on ToxR, as well as on both the *tcpA* and *toxT* promoters. As shown in Fig. 4B, the $toxT_{\Delta pro}$ strain could be complemented for TcpA production by a plasmid encoding an IPTG-inducible *toxT* gene, indicating that the *tcpA* promoter in this background remains responsive to ToxT. In contrast, expression of ToxT in the $tcpA_{\Delta pro}$ strain did not restore TcpA production (Fig. 4C), thereby confirming that the deletion removed sites critical for ToxT-dependent promoter activation.

TcpP and TcpH are required for *toxT* transcription and coordinate regulation. Two other gene products encoded in the *tcp* gene cluster, TcpP and TcpH, have been implicated in coordinate regulation in *V. cholerae* through their effects on *toxT* transcription (3, 13, 20). To characterize the role of TcpP and TcpH in the context of the regulatory loop model analyzed above, mutant strains carrying deletions in these genes (Δ*tcpP* and Δ*tcpH*) were constructed and their RNAs were used in primer extension reactions with a radiolabeled *toxT* primer. Deletion of the *tcpP* gene resulted in complete abolishment of both *toxT*-specific primer extension products (Fig. 5A) and reduction of TcpA to the background level (data not shown). Both *toxT* transcription and TcpA production were restored upon introduction of a *tcpPH*-encoding plasmid into the *tcpP* mutant.

In contrast to the abolishment of *toxT* transcription in the *tcpP* mutant, the *tcpH* mutant strain synthesized detectable *toxT* mRNA, although the pattern of transcription was altered relative to the wild-type pattern (Fig. 5B). This intermediate phenotype for *toxT* transcription caused by the *tcpH* lesion was also seen in toxin production measured by GM_1-ganglioside ELISA. As seen in Table 2, the Δ*tcpP* mutant strain synthesized a barely detectable level of CTX, whereas the Δ*tcpH* mutant strain made a low but easily detectable level of CTX. These data suggest that TcpP is an absolute requirement for *toxT* transcription and subsequent expression of CTX and TCP, while TcpH is required for production and/or maintenance of wild-type levels of *toxT* transcription without being strictly required for transcription activation.

DISCUSSION

Previous studies showed that virulence gene expression in *V. cholerae* is controlled through a regulatory cascade by the ToxR-ToxT system (7, 9, 15). According to this model, activation of *toxT* expression requires ToxR, and gene fusion studies and electrophoretic mobility shift assays showed that there is a ToxR binding site between −73 and −114 relative to the *toxT* transcript initiation site that is required for activation of *toxT* transcription (14). Virulence gene expression is activated by ToxT, whose carboxyl terminus is similar to the DNA-binding domain of the AraC transcription activator of *E. coli* and *Salmonella typhimurium* (15); thus, it likely acts in a manner similar to that of AraC.

Previous work suggested that the ToxR-dependent promoter is not the only source of *toxT* mRNA (2, 14). This conclusion rests on the observation that insertions in the *tcpA* gene have

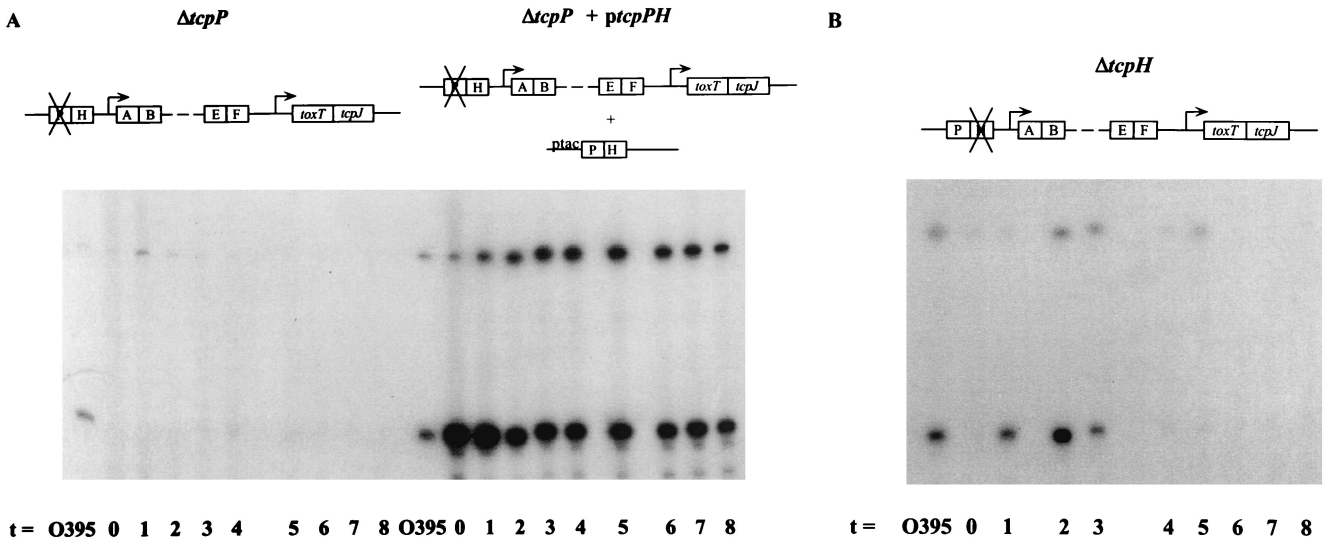


FIG. 5. RNA primer extension analyses of *V. cholerae* mutant strains Δ*tcpP*/pMMB66EH and Δ*tcpP*/p*tcpPH* (A) and mutant strain Δ*tcpH* (B). Samples were collected at 1-h intervals after back dilution of a culture grown overnight in LB medium at 30°C 1:100 into fresh LB medium. IPTG (1 mM) was used to induce transcription in strains carrying plasmids. A radiolabeled *toxT* primer was used. RNA collected from wild-type strain O395 after 2 to 3 h of growth was used as a positive control for all of the mutant strains. p*tcpPH* harbors the *tcpP* and *tcpH* ORFs on low-copy-number plasmid pMMB66EH under the control of the IPTG-inducible *tac* promoter.

a negative effect on toxin production, which suggests that a long *tcp* transcript initiating at the *tcpA* promoter also encodes *toxT*. RNase protection analysis supported this possibility (2). The presence of a transcription terminator between *tcpF* and *toxT*, albeit a relatively weak one compared to a well-characterized λ terminator, indicates that not all transcripts initiating at the *tcpA* promoter would read through to *toxT* (14). While the results presented in this report generally support the autoregulatory loop of transcription in the *tcp* operon proposed by Brown and Taylor (2), there is a slight discrepancy between our results and theirs in the levels of CTX produced by strains with mutations in *tcpA*. The $tcpA_{\Delta pro}$ strain we constructed for this study synthesizes nearly wild-type levels of CTX, with, at most, a twofold decrease compared with the wild type. In contrast, *tcpA* transposon insertion mutants in the study of Brown and Taylor (such as CS2-1 and RT110.21) synthesize approximately 10-fold less CTX than the wild type (2). The basis of this discrepancy is not clear.

The observation that a *toxT-lacZ* fusion is activated to high levels in wild-type *V. cholerae* but that ToxR does not activate *toxT-lacZ* expression in *E. coli* led to the suggestion that ToxR is necessary but not sufficient for activation of *toxT* (14). The TcpP gene product had been implicated in coordinate regulation in the ToxR system by others (20, 37), including Häse and Mekalanos, who most recently demonstrated that a *tcpP* mutant *V. cholerae* strain does not activate expression of a *toxT-lacZ* gene fusion (13). These investigators also showed that overexpressed TcpP activates *toxT* transcription in the absence of ToxR, although wild-type expression of TcpP does not (13). We also note that overexpression of TcpPH in the Δ*tcpP* strain led to *toxT* expression that was sustained and stronger than that observed in the wild type (cf. Fig. 2A and 5A) and a concomitant increase in CTX compared to the wild type (Table 2). This implies that TcpP and TcpH are limiting in this system and, if this is so, may provide evidence for another level of control in the ToxR regulatory system. One hypothesis that might account for the elevated CTX levels when TcpP and TcpH are overexpressed is that TcpP activates *ctxAB* transcription directly. However, overexpression of TcpP and TcpH in the $toxT_{\Delta pro}$ strain did not result in detectable CTX production (data not shown), confirming that TcpP and TcpH mediate their effect on CTX expression solely through the *toxT* promoter. Combined with the fact that ToxR is normally required for *toxT* transcription, we conclude that ToxR and TcpP work together in some way to activate the *toxT* promoter.

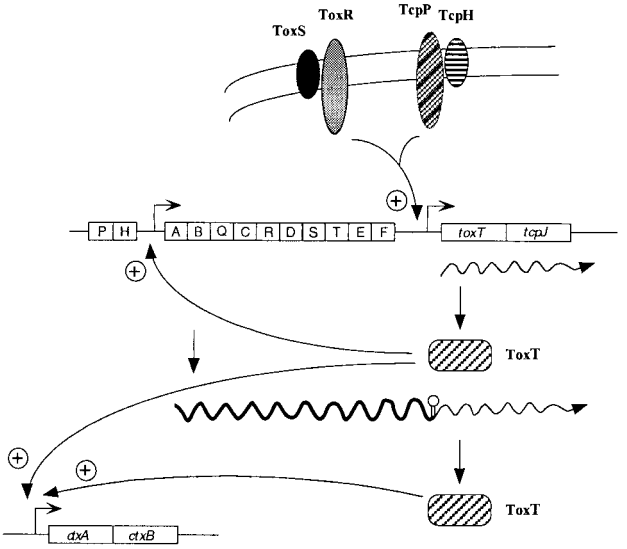


FIG. 6. Model for control of *toxT* transcription and coordinate regulation of virulence in *V. cholerae*. ToxR, ToxS, TcpP, and TcpH in the inner membrane activate *toxT* transcription from the *toxT* promoter. ToxT protein then activates transcription of the *tcpA* promoter, producing more ToxT from the readthrough transcript. ToxT also activates transcription of other virulence genes, including *ctx*, as shown here. The *tcp* gene cluster and the *ctx* operon are shown with each box representing a gene. Symbols: ⌜, promoter; ⇝, transcript of the relevant genes; ⇑ stem-loop structure which is a putative RNA-processing site for the readthrough transcript initiating from the *tcpA* promoter.

These data support the following model for transcription control of *toxT* and coordinate regulation of virulence in *V. cholerae* (Fig. 6). ToxR, ToxS, TcpP, and TcpH in the inner membrane activate *toxT* transcription from the *toxT* promoter, generating the transcript represented by the smaller extension product seen in our experiments. ToxT made from this transcript activates the *tcpA* promoter, producing a long transcript that reads through a weak terminator between *tcpF* and *toxT*, thereby generating another source of *toxT* mRNA. The readthrough transcript may be processed at the site to which the longer primer extension product maps. ToxT from both transcripts is necessary for maximal expression of CTX.

There are alternative hypotheses that would account for our data regarding the two RNA species observed in the primer extension experiments. One is that the larger one originates from a promoter activated by a product of one of the genes coexpressed with *tcpA*. We do not favor this possibility because none of the ORFs downstream of *tcpA* encodes a protein with homology to transcription activators. Another hypothesis is that readthrough transcription could result in the utilization of another upstream *toxT* promoter. Although this has not been ruled out, there are no putative −35 and −10 sequences preceding the site to which the larger *toxT* primer extension product maps.

The fact that two different activators, ToxR and TcpP, are required for priming of the autoregulatory loop leading to *toxT* expression raises questions about how this system evolved. One way to view this issue is to assume that prior to acquisition of the *V. cholerae* pathogenicity island (18) by virulent *V. cholerae*, ToxR controlled only *ompU* expression (as well as that of the ToxR-repressed *ompT* gene). While the island encodes an activator of *toxT*—TcpP—the level of *toxT* activation by TcpP alone in *V. cholerae* may not have been sufficient for a competitive advantage. However, under conditions appropriate for activation of *ompU* by ToxR, perhaps there was a competitive advantage for strains that also expressed CTX and TCP. This would have been a driving force allowing ToxR to take control of the TcpP-dependent activation of *toxT* with subsequent CTX and TCP expression. That TcpP remains a requirement in this system (i.e., that ToxR did not gain complete control over *toxT* transcription) may indicate that its role in *toxT* expression is not limited to transcription activation. One possibility, for example, is that TcpP is an important component of the signaling pathway leading to *toxT* expression.

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