Transcriptional control of *toxT*, a regulatory gene in the ToxR regulon of *Vibrio cholerae*

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Summary

Co-ordinate expression of many virulence genes in *Vibrio cholerae* is under the control of the ToxR and ToxT proteins. These proteins function in a regulatory cascade in which ToxR is required to activate *toxT*, and ToxT activates virulence genes. The precise mechanism for ToxR activation of *toxT* is unknown, but data presented in this report suggest a direct involvement of ToxR. Primer extension and gene fusion analyses identified a ToxR-regulated promoter directly upstream of *toxT*, immediately following a region of inverted repeats capable of terminating transcription. Gel mobility shift studies indicate that ToxR binds DNA within the inverted repeat region, yet preliminary evidence suggests that ToxR binding alone is not sufficient for activation of *toxT*. Possible mechanisms of ToxR-dependent *toxT* expression are discussed.

Introduction

Co-ordinate expression of many virulence genes in the human diarrhoeal pathogen *Vibrio cholerae* is under the control of the ToxR, ToxS and ToxT proteins (DiRita *et al.*, 1991; Higgins *et al.*, 1992; Miller *et al.*, 1987; 1989; Miller and Mekalanos, 1984; Peterson and Mekalanos, 1988; Taylor *et al.*, 1987). ToxR is an inner membrane protein that shares homology with members of the two-component family of transcriptional activators found in various species of bacteria (Miller *et al.*, 1987; Stock *et al.*, 1989). Unlike other members of this family, however, ToxR does not share the characteristic phosphoacceptor domain and is believed to be activated in some manner other than phosphorylation by a sensor kinase. It has been proposed that ToxR is active as a homodimer and that ToxS is required to stabilize ToxR into the dimer

form (DiRita and Mekalanos, 1991). The ToxR protein activates transcription of the genes coding for the cholera toxin *(ctxAB)* by directly binding to a repeated DNA element, TTTTGAT, which is present in three to eight copies immediately upstream of the *ctx* promoter (Miller *et al.*, 1987). In addition to the *ctx* genes, other genes necessary for colonization and full virulence require ToxR for their expression. These include genes for the production of the toxin-coregulated pilus (TCP) (Taylor *et al.*, 1987), and several accessory colonization factor (ACF) genes (Peterson and Mekalanos, 1988; Parsot and Mekalanos, 1992). Furthermore, expression of these virulence genes is under environmental control, as changes in pH, osmolarity and temperature during *in vitro* growth modulate expression of ToxR-dependent genes (DiRita *et al.*, 1991).

Other than the *ctx* operon, ToxR does not directly activate transcription of the virulence genes described above (DiRita *et al.*, 1991). Instead, ToxR mediates its control of these additional virulence genes through the ToxT protein (DiRita *et al.*, 1991; Higgins *et al.*, 1992). ToxT is a member of the AraC family of transcriptional activators (Higgins *et al.*, 1992) and it activates many ToxR-regulated genes, including *tcpA*, *tcpC*, *tcpI* and *ctxAB* (DiRita *et al.*, 1991). Previous work on the ToxR system led to a cascade model in which ToxR-regulated activation of *toxT* transcription results in co-ordinate activation of virulence genes by ToxT in *V. cholerae* (DiRita, 1992; Higgins *et al.*, 1992). This model is supported by the observations that *toxT* specific message is undetectable in *toxR* mutant cells and that *toxT* under control of the constitutive *tet* promoter restores expression of TcpA and Ctx when introduced into a *toxR* mutant *V. cholerae* (DiRita *et al.*, 1991; Higgins *et al.*, 1992). Furthermore, in *toxR* mutant cells expressing *toxT* from the *tet* promoter, virulence gene activation is no longer subject to environmental modulation (DiRita *et al.*, 1991). Therefore, the regulated expression of *toxT* is an important step in controlling virulence in *V. cholerae*. The precise role of ToxR in controlling expression of *toxT* is as yet uncharacterized. Whether ToxR directly activates *toxT* in a manner similar to its activation of *ctxAB*, or does so indirectly through another mechanism is unknown. In this report, we present data that suggest a direct involvement of ToxR in *toxT* activation and characterize *cis*-acting regions involved in the regulated expression of *toxT*.

Results

Mapping of the toxT transcription initiation site

The *toxT* gene resides on the *V. cholerae* chromosome 210 nucleotides downstream of the *tcpF* gene with its stop codon overlapping the start codon for the *tcpJ* gene (Fig. 1; Higgins *et al.*, 1992; Kaufman *et al.*, 1991; Ogierman and Manning, 1992). The repeated ToxR-binding motif TTTTGAT, which is a component of the *ctx* promoter, is not present in the intergenic region between *tcpF* and *toxT*, or within several kilobases upstream of *tcpF* (Higgins *et al.*, 1992; Ogierman *et al.*, 1993). The *tcpF–toxT* intergenic region does contain several inverted repeat elements which have the potential to form secondary stem-loop structures and may play a role in the expression of *toxT* (see below). In order to define the initiation site of *toxT* transcription, primer extension mapping was performed using RNA isolated from *V. cholerae*. Wild-type (O395) and *toxR* mutant (JJM43) classical strains of *V. cholerae* were grown overnight at 30°C in Luria–Bertani (LB) medium with a starting pH of 8.5, a condition that does not favour ToxR-regulated gene expression (DiRita *et al.*, 1991). These were then diluted (1:10) into fresh medium at pH 6.5 and grown at 30°C, conditions that favour ToxR-regulated gene expression, for one hour. RNA was then isolated from cultures and primer extension analysis performed using a primer complementary to the very 5′ portion of the *toxT* open reading frame.

Two predominant primer extension products that mapped within the *tcpF–toxT* intergenic region were identified using RNA from the wild-type strain, whereas only one of these was identified when RNA from the *toxR* mutant was used (Fig. 2). The primer used in the extension analysis was used in a DNA sequencing reaction to determine the precise nucleotide to which each primer extension product maps (indicated by asterisks in Fig. 3). Previous computer analysis of the intergenic region identified a putative prokaryotic promoter element (Higgins *et al.*, 1992) directly upstream of the site that maps closer to the start of the *toxT* gene (Fig. 3). The spacing between the site mapped by primer extension and this element is consistent with the element being a promoter for transcription initiation at the site. However, the −35 portion is not a good match with the consensus prokaryotic sequence (Fig. 3). Poor match to the
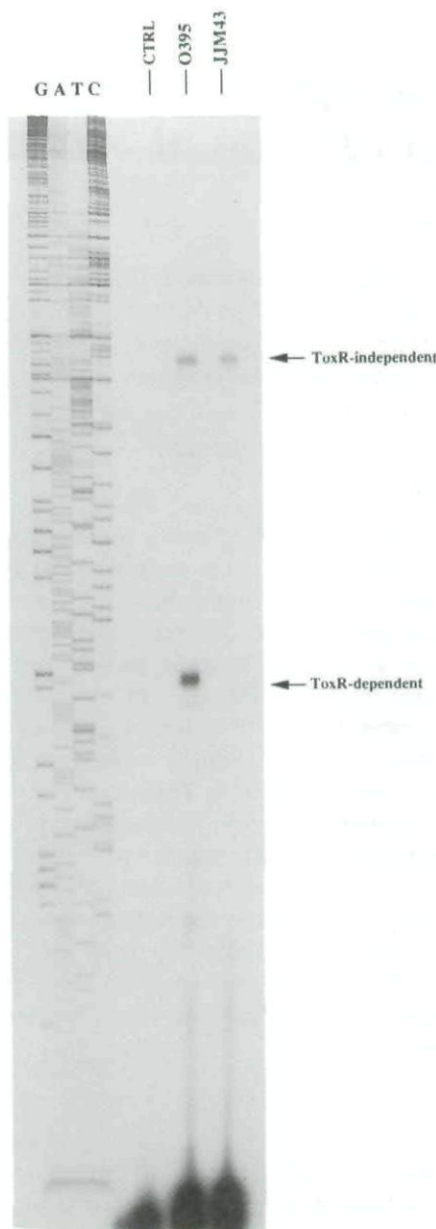

Fig. 2. Primer extension analysis of *toxT* transcripts. RNA was isolated from *V. cholerae* wild type (O395) or *toxR* mutant (JJM43) classical strains after 1 h of growth from shifting overnight cultures grown in LB medium at pH 8.5 to pH 6.5. A radiolabelled primer was incubated with the RNA, and reverse transcriptase was added to produce a DNA copy of the transcript, as described in the *Experimental procedures*. The control lane had no RNA added. The same primer was used to prime dideoxy sequencing products from a DNA template that contained the *tcp* region shown in Fig. 1. The letters above each lane indicate the dideoxynucleotide used to terminate each reaction. The ToxR-dependent and independent extension products are indicated.

.2 kb

Fig. 1. Genetic organization of a portion of the *tcp* region of *V. cholerae*. The organization of the *tcpE, F, J* and *toxT* genes is shown. The arrows indicate the direction of transcription.

consensus −35 element has been noted for promoters that require activators for elevated expression (Raibaud and Schwartz, 1984). Because the primer extension product mapping to this site was not identified using RNA
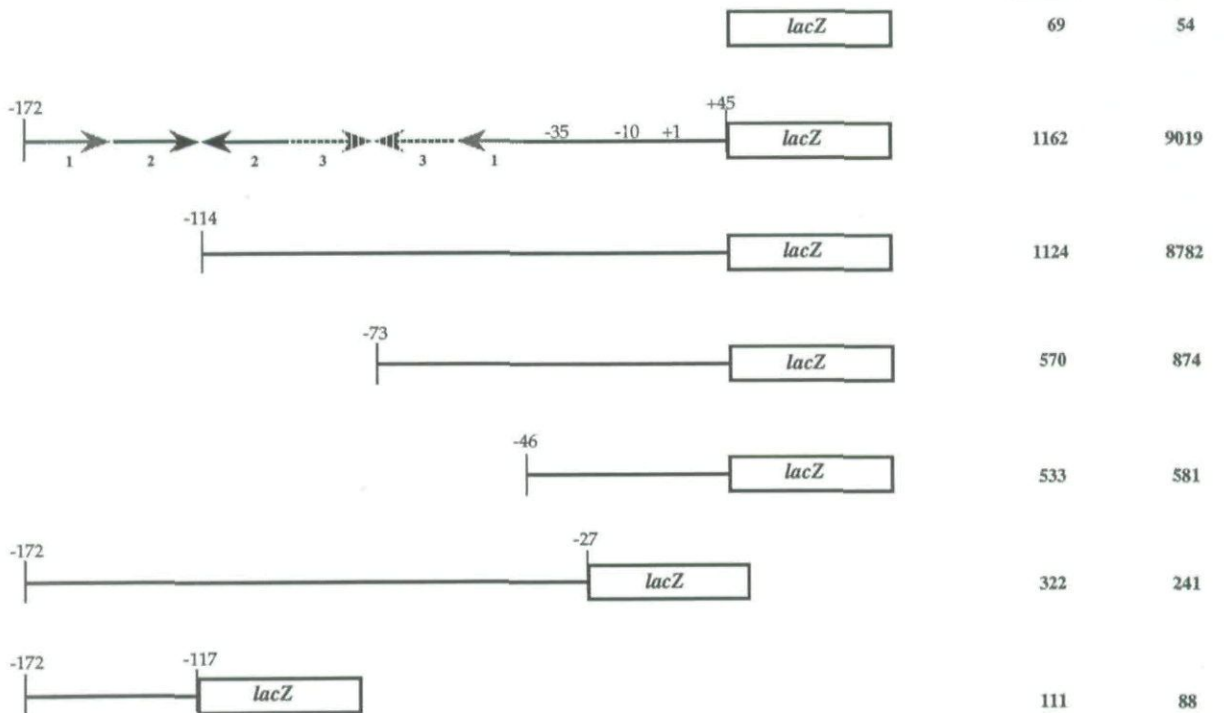
from the *toxR* mutant strain, we conclude that this is a potential ToxR-dependent transcription initiation site. The other primer extension product, which was detected using RNA from both wild-type and *toxR* mutant strains is therefore ToxR-independent. The site to which this ToxR-independent product maps is within one of the inverted repeats noted above and is not preceded by a consensus promoter element directly upstream. Possible sources of this primer extension product will be discussed below, but from the data in Fig. 2, we conclude that a ToxR-regulated promoter is present upstream of the *toxT* gene. Additional analysis indicates that both of the primer extension products shown in Fig. 2 are specific for *toxT* mRNA, since primer extensions with different primers specific for various regions of *toxT* map to the same two sites (data not shown).

Transcription termination within the tcpF–toxT *intergenic region*

We previously speculated that *toxT* expression could be the result of transcription initiating from a promoter upstream of *tcpF*, with the majority of transcripts terminating within the potential stem-loop structures downstream of *tcpF* and a low percentage of the transcripts proceeding into *toxT* (Higgins *et al.*, 1992). Although other investigators have indicated that a potential stem-loop structure following *tcpF* would serve as a Rho-independent transcriptional terminator delineating the TCP gene cluster (Ogierman and Manning, 1992; Ogierman *et al.*, 1993), experiments directly testing the ability of this region to terminate transcription have not been reported. To investigate whether DNA from the *tcpF–toxT* intergenic region can terminate transcription, we used the transcription

Table 1. Transcription termination within the *tcpF–toxT* intergenic region.

Reporter[a]	Units of galactokinase activity[b]	% of control activity[c]
pKL600 (control)	332	100
pB51	61	18.4
ptR2	27	8.1

See Table 3 for description of the reporter plasmids.
a. Plasmid copy number was verified to be similar in all cases.
b. Numbers represent the average of at least three individual experiments.
c. Percent of control activity represents the amount of galactokinase activity relative to the pKL600 reporter with no terminator present.

termination assay vector pKL600 (Cheng *et al.*, 1991). pKL600 is a pBR322-based vector containing a p*lac* promoter followed by a multiple cloning region preceding the gene coding for galactokinase (*galK*). A segment of the intergenic region (between the (^) markings shown in Fig. 3) was cloned into the multiple cloning region of pKL600 yielding plasmid pB51. This portion of the intergenic region was chosen because it contains the inverted repeats, but lacks the putative −10 element upstream of the ToxR-dependent transcription initiation site identified by primer extension. Transcription termination was assayed in *Escherichia coli* and was calculated by comparing the amount of *galK* activity in pB51 to that of the pKL600 vector containing no termination region. The strain with pB51 showed a decrease of at least 80% in the amount of *galK* activity as compared to that of pKL600 (Table 1). This indicates possible transcription termination within the intergenic region upstream of *toxT*. The data also suggest that some transcripts (~20%) may read through to *toxT*. In a control experiment, a plasmid containing the phage lambda tR2 terminator, a strong terminator of

Fig. 3. Nucleotide sequence of the *tcpF–toxT* intergenic region. Sequences within *tcpF* and *toxT* are shown in bold. Arrows above the sequence indicate inverted repeat regions and are arbitrarily numbered as 1, 2, and 3. The putative Shine–Dalgarno (SD) translation initiation sequence and ToxR-dependent promoter sequence are underlined and labelled. The ToxR-dependent and -independent primer extension sites are labelled and indicated with asterisks (*). The extension primer used was complementary in sequence to the very 5′ portion of *toxT* as indicated by the labelled arrow. Nucleotides within and including those underscored by a (^) represent the region tested for the ability to terminate transcription.

Fig. 4. Analysis of *tcpF–toxT* intergenic region fusions in *V. cholerae*. Fusions were made by cloning PCR products harbouring portions of the *tcpF–toxT* intergenic region in front of a promoterless *lacZ* gene on a broad-host-range plasmid. After determining that the PCR product sequence was wild type, the recombinant plasmids were introduced into *V. cholerae*. Beta-galactosidase activity was measured as described in the *Experimental procedures* and is reported in Miller units (Miller, 1972). Δ*toxR* and *toxR⁺* represent the results from fusions in *V. cholerae* strains JJM43 and O395, respectively. Arrows represent the presence of inverted repeats in the DNA sequence and are labelled as in Fig. 3. The ToxR-dependent primer extension product maps to the position labelled +1 on the figure and the positions of the putative −35 and −10 RNA polymerase binding elements are noted. Other numbers on the figure represent positions relative to the +1. The *tcpF* gene coding sequence ends within the upstream inverted repeat number 1.

transcription (Cheng *et al.*, 1991), yielded only 8% of *galK* activity as compared with pKL600 (Table 1). Although we infer from these experiments that the cloned *tcpF–toxT* intergenic DNA contains a transcription termination signal, we cannot rule out the possibility that an RNA processing event is occurring which leads to a decrease in *galK* mRNA.

Identification of a ToxR-regulated toxT *promoter*

The primer extension analysis reported above identified a potential ToxR-dependent transcription initiation site directly upstream of *toxT* within the *tcpF–toxT* intergenic region. In order to localize the ToxR-dependent promoter responsible for *toxT* transcription, fusions were constructed by cloning fragments from the *tcpF–toxT* intergenic region generated by PCR in front of a promoterless *lacZ* gene on a broad-host-range plasmid (pTL61T; Linn and Pierre, 1990). The recombinant plasmids were

introduced into the *V. cholerae* wild type (O395) and *toxR* mutant (JJM43) classical strains and β-galactosidase activity was measured (Fig. 4). A transcriptional fusion containing DNA spanning the end of the *tcpF* gene to just before the start of the *toxT* open reading frame (−172 to +45 relative to the ToxR-dependent start site) expressed eightfold more β-galactosidase activity in wild type than in the *toxR* mutant. A similar result was seen when DNA from −114 to +45 relative to the ToxR-dependent start site was fused to *lacZ*. However, ToxR-regulated β-galactosidase activity was essentially abolished when a fragment containing DNA from −73 to +45 relative to the start site was used. When a fragment missing the −10 portion of the putative promoter (containing DNA from −172 to −27 relative to the ToxR-dependent start site) was fused to *lacZ*, relatively low β-galactosidase activity was expressed in both wild-type and *toxR* mutant strains of *V. cholerae*. Finally, fusion of DNA from −172 to −117 (relative to the start site) to
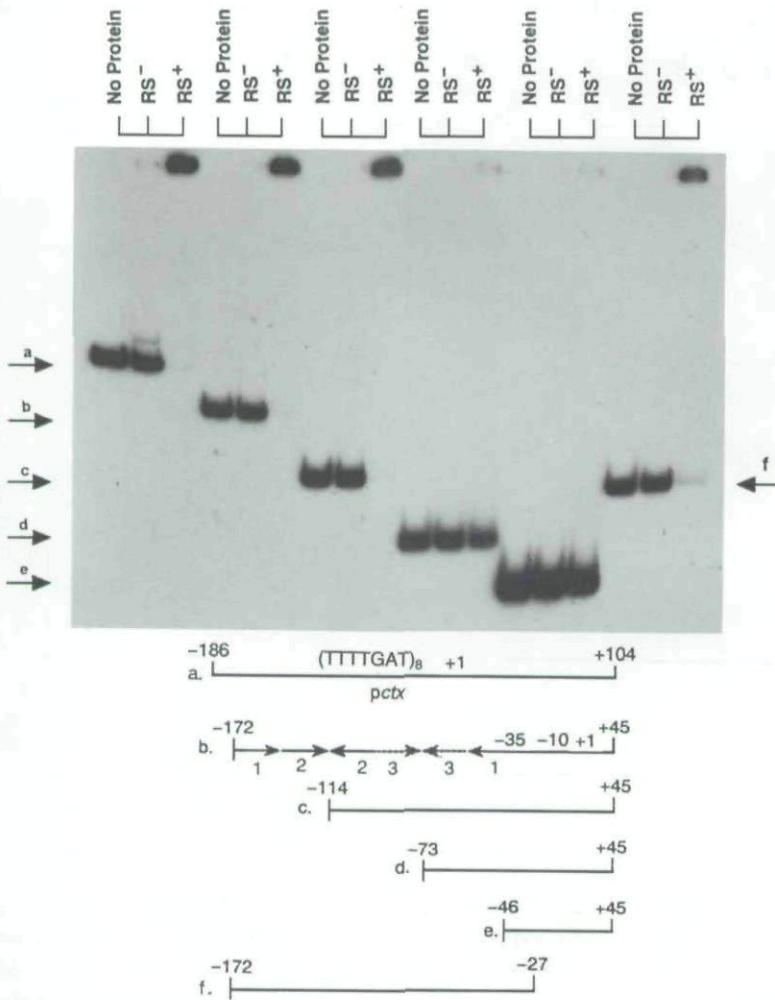
Fig. 5. Ability of ToxR to bind and shift *toxT* promoter DNA. PCR products representing the *ctx* promoter region or various regions of the *toxT* promoter were radiolabelled and used in gel mobility-shift assays with ToxR as described in the *Experimental procedures*. Fragments are labelled a–f and represent the corresponding DNA region depicted below the figure. The *ctx* promoter fragment contained eight copies of the TTTTGAT ToxR-binding sequence. Labels at the top of the figure indicate whether DNA alone (No Protein), membranes without ToxR and ToxS (RS⁻), or membranes containing ToxR and ToxS (RS⁺) were used in the assay.

lacZ, resulted in a low level of β-galactosidase activity that was unaffected by ToxR.

Although conclusions regarding the magnitude of ToxR-dependent activation of *toxT* expression cannot be drawn from these experiments because the reporter gene is on a plasmid, the results strongly suggest that a ToxR-regulated promoter resides upstream of *toxT*. Hence, we conclude that the promoter element identified in Fig. 3 is required for directing *toxT* transcription initiation, presumably at the ToxR-dependent start site mapped by primer extension (Fig. 2), and that a region of DNA between −114 and −73 relative to the ToxR-dependent start site is necessary for ToxR-regulated activation of this promoter.

ToxR binds to toxT *promoter DNA*

ToxR binds to DNA containing at least three copies of the sequence TTTTGAT upstream of the cholera toxin promoter and activates transcription of the cholera toxin genes (Miller *et al.*, 1987). To determine whether or not ToxR-dependent activation of the *toxT* promoter involves direct interaction by ToxR, the ability of membranes containing ToxR to bind *toxT* promoter DNA was assayed in gel mobility-shift experiments (Miller *et al.*, 1987; Ottemann *et al.*, 1992). Total cell membranes were prepared from *E. coli* strain AAEC189 harbouring either the *toxRS* plasmid pVJ21 (Miller *et al.*, 1989) or the vector for pVJ21, pACYC184 (Chang and Cohen, 1978). These membrane preparations were used in DNA gel mobility-shift assays with the PCR fragments used in the promoter studies described above. As shown in Fig. 5, membranes containing ToxR shifted fragments containing DNA from 114 to 172 nucleotides upstream of the ToxR-dependent transcriptional start site. The fragments that are shifted correlate exactly with those that directed ToxR-regulated β-galactosidase activity in *V. cholerae* when fused to *lacZ* (Fig. 4). For example, a DNA fragment
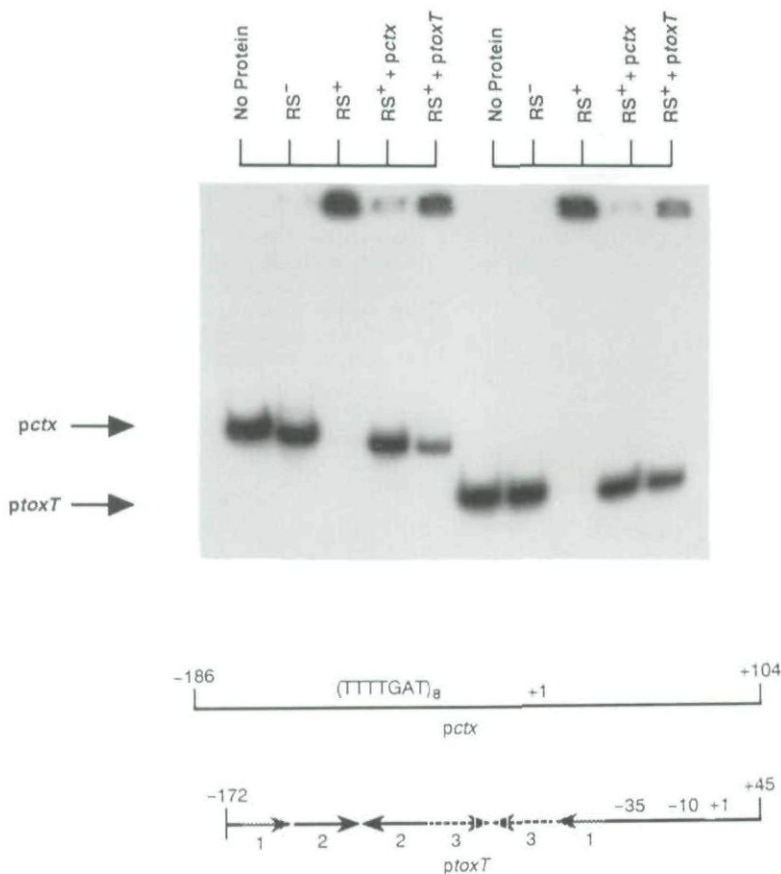
Fig. 6. Ability of *toxT* and *ctx* promoter DNA to compete for ToxR binding. Gel mobility-shift assays were performed as in Fig. 5. +p*ctx* and +p*toxT* represent assays in which an excess amount of unlabelled *ctx* or *toxT* promoter fragment was added to the binding reaction.

with 114 nucleotides of upstream DNA had both ToxR-dependent promoter activity and was bound by membranes containing ToxR, while a fragment with 73 nucleotides of upstream DNA conferred very little ToxR-dependent β-galactosidase activity and was not detectably bound by ToxR-containing membranes. Although the fragment missing the −10 portion of the ToxR-dependent promoter did not exhibit any regulated activity (Fig. 4), it still contains the ToxR-binding region upstream of −73 and consequently was shifted in the binding assay (f in Fig. 5).

ctx *and* toxT *promoter DNA can compete for ToxR binding*

While the ToxR-binding region upstream of the ToxR-dependent *toxT* promoter is rich in secondary structure, partially because of the presence of a potential transcription terminator, it does not contain the repeated TTTTGAT motif required for ToxR binding to the *ctx* promoter. This suggests that ToxR can bind to at least two different DNA primary sequences. To determine if the *ctx* and *toxT* promoters could compete for ToxR binding, competition gel mobility-shift assays were performed.

Radiolabelled *ctx* or *toxT* promoter fragments were

mixed with an excess of unlabelled fragment of the same or opposing promoter and used in the gel mobility-shift assay (Fig. 6). Excess unlabelled DNA containing the *ctx* or *toxT* ToxR-binding regions was able to compete for ToxR binding to labelled fragments containing either promoter. In addition, a DNA fragment of 243 nucleotides from the *V. cholerae tcp* gene cluster that does not have known ToxR-dependent promoter activity was not able to compete for binding to either the *ctx* or *toxT* promoter fragments (data not shown). Thus, ToxR appears to bind specifically to the two different promoters.

ToxR *does not directly activate the* toxT *promoter in* E. coli

ToxR binding upstream of the *ctx* promoter is sufficient to activate *ctx* transcription in *E. coli* (Miller *et al.*, 1987). As ToxR can bind directly upstream of the *toxT* promoter, we wished to determine if ToxR could directly activate transcription of *toxT*. The same *lacZ* fusion plasmids tested in *V. cholerae* were introduced by transformation into *E. coli* strain AAEC189, containing either the *toxRS* plasmid pVJ21 or vector plasmid pACYC184, and β-galactosidase activity was measured (Fig. 7). In the presence of ToxR, fusions harbouring the ToxR-binding portion of the *toxT*
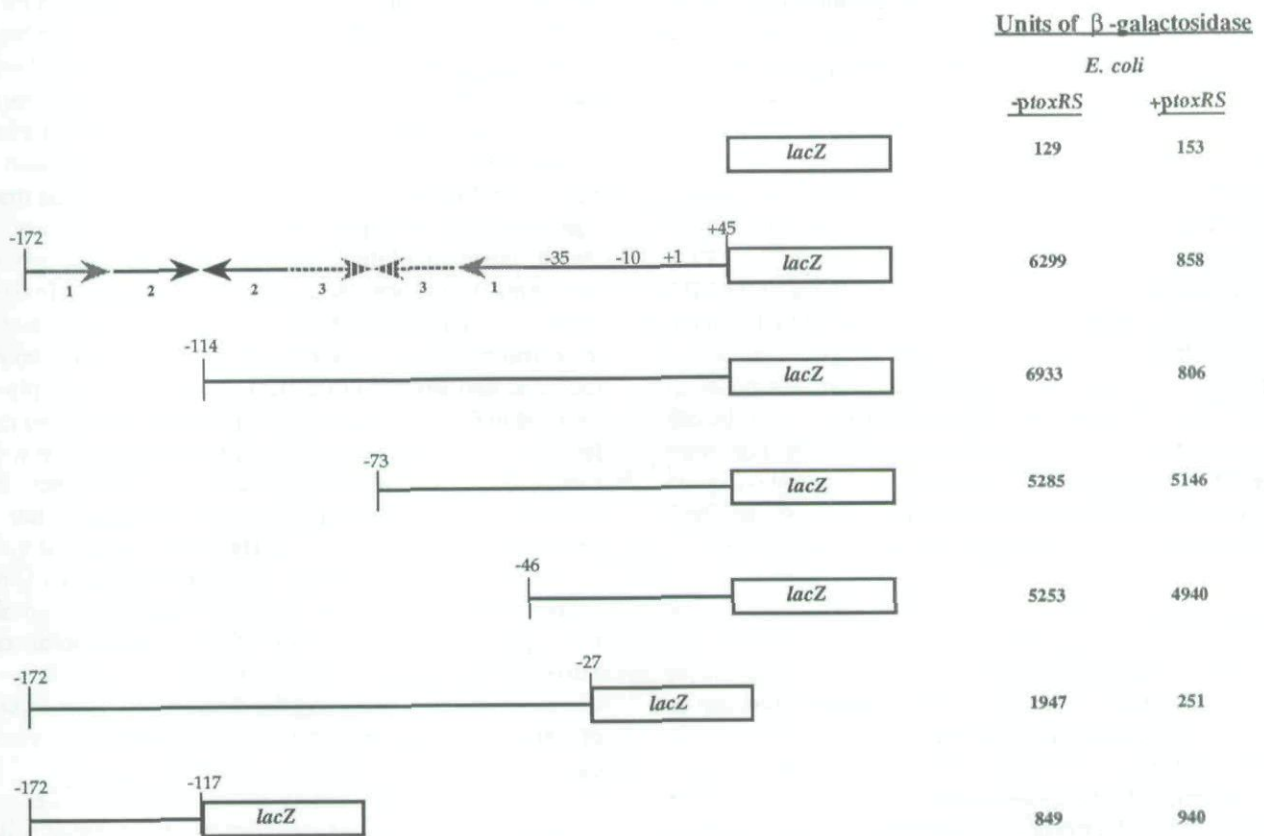
Fig. 7. Analysis of *tcpF–toxT* intergenic region fusions in *E. coli*. Fusions analysed were identical to those described in Fig. 4. −p*toxRS* and +p*toxRS* represent respectively, results from fusions in *E. coli* strain AAEC189 containing plasmid pACYC184 or pVJ21.

promoter (containing DNA from −172 to +45 or −114 to +45 relative to the ToxR-dependent start site) did not show any activation, but instead demonstrated an eightfold decrease in the amount of β-galactosidase activity. This inhibitory effect of ToxR was not observed when *lacZ* fusions containing only 73 or 46 nucleotides of DNA from directly upstream of *toxT* were used; high levels of β-galactosidase activity were expressed from these fusions in both the absence and presence of ToxR. The decrease in β-galactosidase activity observed with the fragment lacking the putative −10 element (containing DNA from −172 to −27 relative to the ToxR-dependent start site) is consistent with our conclusion that the β-galactosidase activity from the fusions is predominantly due to initiation from the ToxR-dependent site shown in Fig. 3. However, this fragment does contain the ToxR-binding region and a similar repression of activity was seen when ToxR was present (Fig. 7). From these observations it appears that ToxR does not directly activate transcription of the *toxT* promoter in *E. coli*. Although the fusion lacking the putative −10 element expresses significant β-galactosidase activity in the absence of ToxR (approximately 2000 units), it is apparent that other

sequences within the DNA fragment are capable of initiating transcription in *E. coli*. This is demonstrated when a fragment harbouring only the DNA between −172 and −117 relative to the ToxR-dependent start site was fused to *lacZ* (Fig. 7). This fragment lacks the complete putative promoter element yet expresses approximately 900 units of β-galactosidase activity in the absence of ToxR. Again, as this fragment does not contain the ToxR-binding region, β-galactosidase activity was unaffected by the presence of ToxR.

Neither ToxR nor ToxT directly activates a chromosomal toxT–lacZ *fusion in* E. coli

To determine if the inability of ToxR to directly activate the *toxT–lacZ* fusions in *E. coli* is an artefact of having the fusions expressed from plasmids, we constructed *E. coli* strain DH92 as described in the *Experimental procedures*. This *E. coli* strain, which has a *toxT–lacZ* gene fusion on the chromosome, was transformed with pVJ21 (*toxRS*⁺) or pACYC184 and assayed for β-galactosidase activity (Table 2). Although the activities were lower than those encoded by the plasmid-based fusions as a result

of the copy number difference, the essential observation remained the same. In the absence of ToxR, strain DH92 expressed 158 units of activity and the level was reduced to 24 units by introducing ToxR. As the *ctx* promoter is activated by both ToxR and ToxT (DiRita *et al.*, 1991), it was of interest to know whether ToxT has an effect on *toxT–lacZ* expression. To this end, we introduced plasmid pMMTT, in which *toxT* is cloned behind an inducible *tac* promoter in the vector pMMB208 (Morales *et al.*, 1991), into the *toxT–lacZ* fusion strain DH92 (Table 2). pMMTT activates a *tcpA–phoA* fusion in *E. coli* 28-fold (unpublished data). However, pMMTT had no significant effect on β-galactosidase activity in DH92. From this observation it would appear that *toxT* expression is not directly autoregulated. When ToxR and ToxT together were tested for activation of the *toxT–lacZ* fusion in strain DH92, a similar repression effect as that seen with ToxR alone was observed (data not shown).

Discussion

Previous studies of the ToxR/ToxT system in *V. cholerae* led to a model in which virulence gene expression is controlled through a regulatory cascade (DiRita, 1992; DiRita *et al.*, 1991; Higgins *et al.*, 1992). According to this model, ToxR directly activates transcription of the genes coding for the cholera toxin (*ctxAB*) and is required for the expression of *toxT*. The ToxT protein, an AraC-like transcriptional activator, in turn directly activates transcription of several other genes required for virulence, such as the TCP and ACF genes, as well as *ctxAB* (DiRita *et al.*, 1991; Higgins *et al.*, 1992). The fact that ToxR-regulated virulence genes can be activated by supplying *toxT* under control of the *tet* or *tac* promoter in a *toxR* mutant of *V. cholerae* (DiRita *et al.*, 1991; P.M. Bruss, and V.J. DiRita, unpublished) indicates that regulated expression of *toxT* is an important step in the signal transduction pathway controlled by ToxR. Determining the precise role played by ToxR in regulating *toxT* expression is critical for understanding this pathway. Whether ToxR is directly or indirectly involved, possibly mediating its control of *toxT* through some other activation factor, has not been explained. Our demonstration of a ToxR-regulated promoter controlling *toxT* transcription in *V. cholerae*, and a ToxR-binding site upstream of *toxT*, imply a direct involvement by ToxR in *toxT* expression.

ToxR-dependent expression of toxT

It is clear from the transcriptional fusion data (Fig. 4) that the ToxR-dependent promoter identified within the *tcpF–toxT* intergenic region is predominantly responsible for regulated expression of *toxT*. This conclusion is based on the approximately eightfold higher amount of

β-galactosidase activity from the *toxT–lacZ* fusions in the *toxR*[+] strain of *V. cholerae,* compared to the levels in the *toxR* mutant, and the abolition of almost all activity when the −10 portion of this promoter is deleted. As is often the case for activated promoters, the −35 portion of this promoter does not conform well to the prokaryotic consensus element (Raibaud and Schwartz, 1984) and thus the presence of a binding site for a transcriptional activator directly upstream of the ToxR-dependent promoter is not surprising. The specific sequence upstream of the ToxR-dependent promoter to which ToxR binds is not yet clear. In activating the *ctx* promoter, ToxR binds a tandemly repeated heptamer, TTTTGAT. This element is not present within DNA bound by ToxR upstream of *toxT*, and in fact this DNA does not contain any direct repeats that we can detect. The DNA between *tcpF* and *toxT* is rich in inverted repeat sequences that are predicted to form stem-loop structures (Fig. 3). It should be noted that the TTTTGAT repeats upstream of *ctx* are actually part of an element of hyphenated dyad symmetry (Mekalanos *et al.*, 1983). Deletion of the TTTTGAT repeats abolished ToxR binding (Miller *et al.*, 1987): it is unclear whether this is the result of disrupting the direct repeats *per se* or of disrupting the symmetrical element composed in part by the repeats.

ToxR alone is sufficient to activate expression of a *ctx–lacZ* fusion in *E. coli* (Miller *et al.*, 1987; Miller and Mekalanos, 1984; Ottemann *et al.*, 1992). However, the data reported here suggest that ToxR binding is not sufficient to allow activation of a *toxT–lacZ* fusion in *E. coli*. The apparent repression by ToxR of *toxT–lacZ* fusions containing the ToxR-binding region in *E. coli* raises questions about the function of ToxR binding within this region. Clearly, ToxR is required for elevated expression of *toxT* in *V. cholerae* and its binding to DNA upstream of the *toxT* promoter suggests a direct involvement. It may simply be the case that the location of the ToxR-binding site on the *toxT* promoter does not allow ToxR to make the proper contact with *E. coli* RNA polymerase for activation of transcription in *E. coli*. This would imply that there are important differences between RNA polymerases of *E. coli* and *V. cholerae*.

It may also be that an additional factor is required for direct activation of the *toxT* promoter by ToxR. The nature of this putative additional requirement is only speculative at present, but candidates for it are DNA topology or other protein factors such as a subunit of the RNA polymerase or a transcriptional activator. Supercoiling and DNA topology can affect the expression of virulence genes in response to environmental stimuli (Dorman *et al.*, 1990; Galan and Curtiss III, 1990; Hulton *et al.*, 1990). In *V. cholerae*, regulated expression of the *acfA* and *acfD* gene promoters is affected by whether reporter gene fusions are chromosomally encoded or

Table 2. Expression of a *toxT–lacZ* chromosomal fusion in *E. coli.*

Effector[a]	Units of β-galactosidase[b]
pACYC184 (−*toxRS*)	158
pVJ21 (+*toxRS*)	24
pMMB208 (−*toxT*)	148
pMMTT (+*toxT*)	159

a. See Table 3 for a description of the effector plasmids.
b. Beta-galactosidase activities are expressed as Miller units (Miller, 1972) and represent the average of a least three individual experiments.

plasmid encoded (Parsot and Mekalanos, 1992). None of the data presented in this report suggests that DNA topology plays any significant role in *toxT* expression. With regard to protein factors, preliminary data indicate that ToxT does not play a role in regulating its own expression, as is evident from the lack of any significant effect of ToxT on the expression of the *toxT–lacZ* chromosomal fusion in *E. coli* (Table 2). In different bacterial species, alternate RNA polymerase sigma factors are required for expression of regulated promoters (Akerley and Miller, 1993; Kenney and Moran, 1987; Straus *et al.*, 1987), and perhaps a *Vibrio* sigma factor is required for *toxT* transcription. Another possibility is that a second transcriptional activator operates at the *toxT* promoter in conjunction with ToxR. We are currently investigating whether additional *V. cholerae* protein factors are required for the activation of *toxT*.

Does transcriptional read-through play a role in toxT expression?

The finding that *toxT* resides within the cluster of genes coding for the toxin coregulated pilus (TCP; Higgins *et al.*, 1992; Ogierman and Manning, 1992), with many of these genes having been shown to require ToxT for expression, led us to propose that ToxR regulates *toxT* expression by activating transcription that initiates upstream of *tcpF* and reads through to *toxT* (Higgins *et al.*, 1992). Although the data presented here clearly indicate that there is a ToxR-dependent promoter directly upstream of *toxT*, they do not rule out the possibility that additional *toxT* transcription occurs as a result of the transcription apparatus reading through the *tcpF–toxT* intergenic region. Such readthrough control of a transcriptional regulator has been proposed for expression of the *prfA* gene of *Listeria monocytogenes* (Camilli *et al.*, 1993).

Identification of a *toxT*-specific primer extension product in RNA from *toxR* mutant *V. cholerae* (Fig. 2) lends the best support to the hypothesis of a readthrough control mechanism as a component of *toxT* expression. This product may be derived from a longer transcript initiating further upstream, given that DNA upstream of the site to which it maps does not have obvious promoter elements. This ToxR-independent site is within one of the inverted

repeat elements potentially forming a stem-loop structure in the RNA (Fig. 3). Secondary structures in RNA are known to be sites for RNA processing events and can function as targets for endoribonucleases (Belasco and Higgins, 1988). Perhaps the ToxR-independent primer extension product actually maps to the 5′ end of an RNA species generated by cleavage of a longer RNA transcript within a structure involving the inverted repeat elements. Another possibility is that secondary structure in the RNA, due to the inverted repeats, might prevent the reverse transcriptase from proceeding through and the primer extension product marks a premature termination in the extension reaction. Secondary structures have been shown to cause reverse transcriptase to pause or stop, resulting in less than full-length primer extension products (Boorstein and Craig, 1989). The speculation that premature termination of the reverse transcriptase or processing of the RNA transcript could occur at a stem-loop structure is consistent with the presence of a minor primer extension product between the ToxR-dependent and independent products on the gel shown in Fig. 2. The 3′ end of this product maps to a site within the inverted repeat labelled 3 in Fig. 3. Because of the high percentage of A and T residues, this predicted stem-loop structure would be significantly less stable than the one directly upstream. Hence, it is expected that the role this putative structure might play in generating a primer extension product with a 3′ end at this position would be minor owing to its weaker predicted stability compared with the one upstream, thereby resulting in the fainter signal that was observed from this product.

Another observation supporting transcriptional read-through as a mechanism of *toxT* expression is our demonstration that the terminator between *tcpF* and *toxT* allows downstream transcription to occur, at least when assayed in *E. coli* (Table 1). This result, as it stands, suggests that a small amount of readthrough transcription from upstream may generate *toxT* mRNA in *V. cholerae*, assuming a mechanism for readthrough transcription exists in *Vibrio*. Difficulties arising in measuring galactokinase activity precluded us from testing these plasmids in *V. cholerae*. We have also observed that ToxT activity in *E. coli* from a plasmid encoding *tcpF* and *toxT* is dependent on a plasmid promoter upstream of *tcpF* (Higgins *et al.*, 1992), which suggests that readthrough does occur and can generate biologically significant levels of ToxT. A definitive demonstration of readthrough transcription of *toxT* in *V. cholerae* requires further experiments. Regardless of how the ToxR-independent primer extension product described in this work is generated, the role of this transcript in virulence gene regulation is questionable given that it was identified in RNA from the *toxR* mutant JJM43 (Fig. 2), in which the ToxR regulon is expressed poorly, if at all.

Table 3. Strains and plasmids used in this work.

Strain/Plasmid	Description	Reference/Source
Strain		
V. cholerae		
O395	Str^R	Laboratory collection
JJM43	O395 Δ*ctxA1* Δ*toxR43*	Herrington *et al.* (1988)
E.coli		
K37	*galK*, Str^R	NIH collection
AAEC189	F$^-$ Δ*fim recA supE44 hsdR17 mcrA mcrB endA1 thi-1* Δ(*argF–lac*)205(U169) λ$^-$	Blomfield *et al.* (1991)
TE2680	F$^-$λ$^-$IN(*rrnD–rrnE*)1 Δ(*lac*)X74 *rpsL galK2 recD1903* ::Tn*10d*-Tet *trpDC700* ::*putPA1303* ::[Kan^S-Cam^R-*lac*]	Elliott (1992)
DH92	TE2680 [Kan^R-p*toxT-lacZ*]	This work
Plasmid		
pKL600	pBR322-based transcription termination test vector; Ap^R	Cheng *et al.* (1991)
pB51	pKL600 with *tcpF–toxT* intergenic region insert; Ap^R	This work
ptR2	pKL600 with λ tR2 terminator insert; Ap^R	Cheng *et al.* (1991)
pTL61T	*lacZ* transcriptional fusion vector; Ap^R	Linn and Pierre (1990)
pACYC184	Cloning vector; Tc^R Cam^R	Chang and Cohen (1978)
pVJ21	pACYC184 *tc* ::*toxRS*	Miller *et al.* (1989)
pMMB208	cloning vector; Cam^R	Morales *et al.* (1991)
pMMTT	pMMB208 with *toxT* insert	This work
pRS551	*lacZ* transcriptional fusion vector; Ap^R Kan^R	Simons *et al.* (1987)
pRSI2	pRS551 p*toxT–lacZ*	This work

Str^R, streptomycin resistant; Ap^R, ampicillin resistant; Tc^R, tetracycline resistant; Kan^R, kanamycin resistant; Cam^R, chloramphenicol resistant.

The precise mechanism for ToxR-regulated expression of *toxT* remains to be elucidated, but the identification of a ToxR-regulated promoter upstream of *toxT* and a previously unidentified ToxR-binding site suggest a direct involvement of ToxR. The fact that the ToxR-binding sequence for *toxT* is completely different from that of *ctxAB* brings up interesting questions concerning the nature of ToxR–DNA interactions. Future studies in this system will give us a clearer understanding of the precise mechanism of ToxR-regulated *toxT* expression and therefore of virulence regulation in *V. cholerae*, and will also provide answers to more fundamental questions of DNA–protein interactions.

Experimental procedures

Bacterial strains and plasmids

The *V. cholerae* and *E. coli* strains and plasmids used in this work are listed in Table 3. Strains were grown in LB medium (Miller, 1972) at 30°C. Strains were maintained at −70°C in LB medium plus 20% glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; tetracycline, 12.5 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; chloramphenicol, 50 μg ml^{-1} and streptomycin, 100 μg ml^{-1}. Transfer of plasmid DNA to *V. cholerae* was carried out by electroporation, and to *E. coli* by transformation using standard protocols (Sambrook *et al.*, 1989).

DNA manipulations

Plasmid DNA was purified by using QIAGEN columns (QIAGEN, Inc.). PCR products containing various portions of the *tcpF–toxT* intergenic region were generated using Vent$_R$ DNA polymerase using the manufacturer's specified procedure (New England Biolabs). PCR reaction templates were either plasmid pDH8 (Higgins *et al.*, 1992) containing a portion of the TCP gene cluster including the complete *tcpF* and *toxT* genes from *V. cholerae* strain O395, or plasmid pJM17 (Mekalanos *et al.*, 1983) containing the *ctxAB* promoter region harbouring eight copies of the TTTTGAT ToxR-binding sequence. When necessary, synthesized primers contained added recognition sequences for restriction endonucleases to facilitate orientation-directed cloning. PCR products were purified by agarose gel electrophoresis followed by gel extraction using the QIAEX gel extraction system (QIAGEN, Inc.). Cloning procedures were carried out using standard protocols (Sambrook *et al.*, 1989). Double-stranded sequencing of plasmids was performed as previously reported (Higgins *et al.*, 1992). The *ctxAB* promoter fragment was sequenced by using the fmol PCR sequencing kit (Promega Biotech).

Primer extension

RNA was isolated from *V. cholerae* strains by the hot-phenol method (DiRita *et al.*, 1991). Ten picamoles of primer   5′-CATTAGTTTGAAAAGATTTTTTCCCAATCAT-3′

was end-labelled using $50\,\mu\mathrm{Ci}$ of $[\gamma\text{-}^{32}\mathrm{P}]$-ATP ($>3000\,\mathrm{Ci}$ mmol^{-1}) and phage T4 polynucleotide kinase (BRL) as described (Sambrook *et al.*, 1989). Approximately 0.2 pmol of labelled primer was added to $20\,\mu\mathrm{g}$ of RNA. Two microlitres of $5\times$ hybridization buffer ($0.5\,\mathrm{M}$ KCl, $0.25\,\mathrm{M}$ Tris HCl, pH 8.3) and $\mathrm{dH_2O}$ to $10\,\mu\mathrm{l}$ was added. Reaction mixtures were incubated at $95°\mathrm{C}$ for 1 min, $55°\mathrm{C}$ for 2 min, and then on ice for 15 min to allow annealing of primer to template. Five microlitres of the annealed reaction mixtures were added to tubes containing $1\,\mu\mathrm{l}$ of $2.5\,\mathrm{mM}$ dGTP, dATP, dTTP, and dCTP mix; $1\,\mu\mathrm{l}$ of $0.1\,\mathrm{M}$ dithiothreitol, $2\,\mu\mathrm{l}$ of reverse transcriptase buffer ($0.25\,\mathrm{M}$ Tris HCl, pH 8.3, $0.375\,\mathrm{M}$ KCl, $15\,\mathrm{mM}$ $\mathrm{MgCl_2}$, $2\,\mathrm{U}$ of RNasin (Promega Biotech) per $\mu\mathrm{l}$), and $1\,\mu\mathrm{l}$ ($200\,\mathrm{U}$) of Superscript reverse transcriptase (BRL). Reaction mixtures were incubated at $44°\mathrm{C}$ for 45 min, and then $5\,\mu\mathrm{l}$ of Sequenase stop buffer (United States Biochemical) was added to terminate the reactions. Extended reaction mixtures were heated to $95°\mathrm{C}$ for 3 min and $5\,\mu\mathrm{l}$ resolved by electrophoresis in 5% denaturing polyacrylamide gels and visualized by autoradiography following standard protocols (Sambrook *et al.*, 1989).

Transcription termination analysis

A PCR product harbouring the portion of the *tcpF–toxT* intergenic region indicated in the legend to Fig. 3, with flanking *Bam*HI and *Xba*I restriction site sequences, was generated and subcloned into *Bam*HI–*Xba*I-digested pKL600 to create pB51. After verifying the intergenic sequence to be wild type, pB51 DNA was transformed into *E. coli* strain K37 and galactokinase activity measured as described (Adhya and Miller, 1979) with the exception that cells were grown in LB medium without the addition of IPTG.

Transcriptional fusion analysis

PCR products harbouring various portions of the *tcpF–toxT* intergenic region were generated with flanking *Xba*I and *Bam*HI sites and subcloned into *Xba*I–*Bam*HI-digested pTL61T. After verifying the intergenic sequences to be wild type, plasmid DNA was transferred into *V. cholerae* strains or *E. coli* strain AAEC189. The *E. coli* strain also contained either the pACYC184 vector or pVJ21 (*toxR*$^+$*S*$^+$). *V. cholerae* cells harbouring plasmids were grown overnight in LB medium with a starting pH of 8.5 at $30°\mathrm{C}$ then diluted 1:50 in fresh LB medium pH 6.5 and grown for 3 h at $30°\mathrm{C}$. Cells were harvested and β-galactosidase activity measured as previously described (Miller, 1972). There is no observable effect of pH on ToxR-regulated gene expression in *E. coli*, so assays for *E. coli* constructs were carried out as in *V. cholerae* except the media starting pH was always 6.5.

Preparation of membrane fractions containing ToxR and ToxS

E. coli membrane fractions containing ToxR and ToxS were prepared as previously described (Miller *et al.*, 1987), except that strain AAEC189 harbouring the plasmid pACYC184 or pVJ21 was used as the source of the membranes. Total protein concentration was determined by reading the optical density at 280 nm (OD_{280}) of membrane fractions.

DNA gel mobility assays

Retardation of radioactively labelled DNA fragments was performed as described previously (Miller *et al.*, 1987; Ottemann *et al.*, 1992) with the following modifications. Gel-extracted PCR products, having been subcloned and the sequences verified, were end-labelled using $[\gamma\text{-}^{32}\mathrm{P}]$-ATP ($>3000\,\mathrm{Ci}\,\mathrm{mmol}^{-1}$) and phage T4 polynucleotide kinase (BRL) (Sambrook *et al.*, 1989). Assays were performed in $20\,\mu\mathrm{l}$ volumes with membrane fractions containing a final protein concentration of $500\,\mu\mathrm{g}\,\mathrm{ml}^{-1}$ and including $12.5\,\mu\mathrm{g}\,\mathrm{ml}^{-1}$ sheared salmon sperm DNA. Competition assays were performed similarly with the addition of a 10-fold mass excess of unlabelled PCR-generated competitor DNA to the binding reaction.

Construction of a toxT–lacZ chromosomal fusion in E. coli

The *toxT–lacZ* chromosomal fusion strain DH92 was constructed using the method of Elliott (1992). Briefly, a PCR product, containing DNA from -172 to $+45$ relative to the ToxR-dependent *toxT* transcription initiation site (Fig. 3), was generated with flanking *Eco*RI and *Bam*HI restriction endonuclease sites. This fragment was subcloned into *Eco*RI–*Bam*HI-digested pRS551 (Simons *et al.*, 1987) yielding plasmid pRSI2. After verifying the sequence was wild type, pRSI2 DNA was linearized by digesting with *Xho*I. Linear DNA was transformed into *E. coli* strain TE2680 and cells then selected for kanamycin resistance (Kan^R) and screened for ampicillin sensitivity (Ap^S) and chloramphenicol sensitivity (Cam^S), which indicate recombination of the fusion on to the chromosome. A Kan^R, Cam^S, Ap^S clone was designated as strain DH92. Plasmids containing *toxR* or *toxT* were separately transformed into DH92 and β-galactosidase activity measured as for *V. cholerae* strains, with the following exception: after shifting to pH 6.5 medium, and growing for 1.5 h, IPTG was added to a final concentration of 0.25 mM to cultures with the pMM series plasmids and these were allowed to grow an additional 1.5 h before β-galactosidase activity was measured.

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