Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in *Vibrio cholerae*

J. Adam Crawford,[1†] **Eric S. Krukonis**[1] **and Victor J. DiRita**[1,2*]

[1]*Department of Microbiology and Immunology, and* [2]*Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI 48109, USA.*

Summary

ToxR is a bitopic membrane protein that controls virulence gene expression in *Vibrio cholerae*. Its cytoplasmic domain is homologous to the winged helix–turn–helix ('winged helix') DNA-binding/transcription activation domain found in a variety of prokaryotic and eukaryotic regulators, whereas its periplasmic domain is of ill-defined function. Several genes in *V. cholerae* are regulated by ToxR, but by apparently different mechanisms. Whereas ToxR directly controls the transcription of genes encoding two outer membrane proteins, OmpU and OmpT, it co-operates with a second membrane-localized transcription factor called TcpP to activate transcription of the gene encoding ToxT, which regulates transcription of cholera toxin (*ctxAB*) and the toxin-co-regulated pilus (*tcp*). To determine the requirements for gene activation by ToxR, different domains of the protein were analysed for their ability to control expression of *toxT*, *ompU* and *ompT*. Soluble forms of the cytoplasmic winged-helix domain regulated *ompU* and *ompT* gene expression properly but did not activate *toxT* transcription. Membrane localization of the winged helix was sufficient for both *omp* gene regulation and TcpP-dependent *toxT* transcription, irrespective of the type of periplasmic domain or even the presence of a periplasmic domain. These results suggest that (i) the major function for membrane localization of ToxR is for its winged-helix domain to co-operate with TcpP to activate transcription; (ii) the periplasmic domain of ToxR is not required for TcpP-dependent activation of *toxT* transcription; and (iii) membrane localization is not a strict requirement for DNA binding and transcription activation by ToxR.

Introduction

Vibrio cholerae is responsible for cholera, a disease characterized by profuse, watery diarrhoea induced by the action of the cholera toxin (CT). CT is an ADP-ribosylating enzyme that causes cAMP accumulation and resultant loss of ions and water from enterocytes into the intestinal lumen (reviewed by Kaper *et al.*, 1995). Another virulence factor of critical importance in the pathogenesis of cholera is the toxin-co-regulated pilus (TCP; Taylor *et al.*, 1987), required for host colonization through an undefined mechanism (Herrington *et al.*, 1988).

The ToxR protein regulates expression of the genes encoding CT and TCP. ToxR defines a class of unusual transcription regulators that, although localized to the cytoplasmic membrane, regulate gene expression by binding DNA and activating transcription (Miller *et al.*, 1987). Other membrane-localized activators like ToxR have been identified in *Vibrio cholerae* (TcpP and CadA) (Häse and Mekalanos, 1998; Merrell and Camilli, 2000) and in other *Vibrio* species (Lin *et al.*, 1993; Reich and Schoolnik, 1994) as well as in a variety of Gram-negative organisms including *Escherichia coli* (Neely *et al.*, 1994), *Salmonella typhimurium* (Blanc-Potard *et al.*, 1999), *Yersinia* spp. (Yang and Isberg, 1997) and *Photobacterium* (Welch and Bartlett, 1998). They all share a common bitopic arrangement with a cytoplasmic amino-terminus and a periplasmic carboxy-terminus separated by a short transmembrane stretch of hydrophobic amino acids. They often work in conjunction with an effector protein that is predominantly periplasmic but localizes to the inner membrane by virtue of a hydrophobic amino-terminal sequence. The effector protein for ToxR is called ToxS, and it is hypothesized that, through periplasmic interaction with ToxR, ToxS may either control ToxR stability or provide a regulatory role for ToxR function (Miller *et al.*, 1989; DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994; Pfau and Taylor, 1998).

Much of the cytoplasmic amino-terminus of ToxR is homologous to a family of bacterial transcription activators

typified by the OmpR protein, a regulator of porin gene expression in *E. coli*. The DNA-binding/transcription activation domain of OmpR is a winged helix–turn–helix (HTH), which is defined by an HTH motif, followed by a wing composed of two β-strands, themselves separated by a loop (Martínez-Hackert and Stock, 1997a,b). In these motifs, the second helix and the wing are thought to provide DNA recognition domains (Schultz *et al.*, 1991; Martínez-Hackert and Stock, 1997b). Specific residues conserved among members of the OmpR family of winged-helix activators, including ToxR, are required for DNA binding (Ottemann *et al.*, 1992), but the subsequent steps in transcription activation have not been determined. Based on the results of a variety of genetic experiments, activation of transcription by OmpR apparently requires interaction of the loop between the two helices of the HTH domain (the α-loop) with the carboxyl-terminal portion of the α-subunit of RNA polymerase (RpoA; Slauch *et al.*, 1991; Russo and Silhavy, 1992; Russo *et al.*, 1993). A variety of transcription factors has been shown to interact with the C-terminal domain of RpoA, and different factors require different residues in RpoA for this activity (Ishihama, 1992).

The process by which ToxR activates transcription is under investigation in order to clarify its role in *V. cholerae* virulence and from an interest in defining the mechanism of action of the unusual class of activator protein that it represents. To regulate CT and TCP expression, ToxR works with another protein called TcpP, which, like ToxR, is membrane localized, has a cytoplasmic domain similar to the DNA-binding/activation domain of OmpR and requires a membrane-anchored effector protein, called TcpH (Carroll *et al.*, 1997; Häse and Mekalanos, 1998). The current model for the mechanism of TcpP and ToxR function in controlling virulence gene expression is that they co-operate to activate expression of the *toxT* gene (Häse and Mekalanos, 1998; Krukonis *et al.*, 2000), the product of which, ToxT, is the direct activator of *ctx* and *tcp* transcription (DiRita *et al.*, 1991; Yu and DiRita, 1999; 2002). An additional step involved in the ToxR/TcpP/ToxT regulatory cascade involves AphA and AphB (activator of *tcpP* and *tcpH* expression), which activate transcription of the *tcpPH* operon in response to environmental signals (Skorupski and Taylor, 1999; Kovacikova and Skorupski, 1999; 2000; 2001; 2002). Finally, a recent report demonstrated that quorum sensing controls the ToxR regulon (Zhu *et al.*, 2002).

Independently of TcpP and TcpH, ToxR and ToxS regulate the production of two major outer membrane porins in *V. cholerae*, OmpU and OmpT. Production of these proteins is reciprocal, in that wild-type cells expressing ToxR produce OmpU, but not OmpT, and mutant cells lacking ToxR produce OmpT, but not OmpU (Miller and Mekalanos, 1988), suggesting that ToxR is an activator of

ompU and a repressor of *ompT*. ToxR-mediated regulation of OmpU and OmpT is critical for bile resistance and intestinal colonization (Provenzano and Klose, 2000; Provenzano *et al.*, 2000; 2001; Wibbenmeyer *et al.*, 2002) and for resistance to organic acids (Merrell *et al.*, 2001). ToxR may also directly activate *ctxAB* transcription independently of TcpP and ToxT, based on the ability of ToxR to activate a *ctx–lacZ* fusion in *E. coli* (Miller *et al.*, 1987; 1989), but this activation event was not detected in *V. cholerae* during *in vitro* growth (Champion *et al.*, 1997). However, ToxR may directly activate the *ctxAB* promoter within the host (Lee *et al.*, 1999).

Study of ToxR-dependent activation of *ompU* and *toxT* and repression of *ompT* has led us to the conclusion that ToxR activates gene expression in different ways at each promoter. In the case of *ompU* activation, DNA binding by ToxR at specific elements within the *ompU* promoter results in transcription activation apparently without the need for co-activators other than ToxS and RNA polymerase (Crawford *et al.*, 1998). To repress *ompT* transcription, ToxR binds to a region that overlaps the *ompT* promoter and a putative CRP binding site, which interferes with CRP-mediated activation of *ompT* transcription (Li *et al.*, 2000; 2002). For *toxT* activation, although ToxR binds to a distinct region within the *toxT* promoter (Higgins *et al.*, 1992; Higgins and DiRita, 1994; 1996; Krukonis *et al.*, 2000; Li *et al.*, 2000), TcpP is also required, and its role in activation is clearly different from that of ToxR, as overexpression of TcpP obviates the requirement for ToxR (Häse and Mekalanos, 1998; Murley *et al.*, 1999; Krukonis *et al.*, 2000), although the converse is not true (Higgins and DiRita, 1994).

In this study, we investigated the structure and localization requirements for ToxR to regulate the expression of *ompU*, *ompT* and *toxT*. Our results show that TcpP-dependent activation of *toxT* requires that the winged-helix domain of ToxR be membrane localized and that, beyond the requirement for having the DNA-binding domain of ToxR localized to the membrane, other domains on the protein are not required for *toxT* transcription. In contrast, regulation of *ompU* and *ompT* does not require membrane localization of the ToxR winged-helix domain, suggesting that the domain remains capable of binding DNA and interacting with the transcription apparatus when not associated with the membrane.

Results

Construction of mutant alleles encoding different domains of ToxR

To investigate the domain requirements for ToxR to activate gene expression, we constructed a variety of *toxR* alleles that encode different domains of the protein

(Fig. 1). The protein encoded by *toxR*cyt-1 has the first 114 amino acids of ToxR, representing only the OmpR-homologous winged-helix domain (Martínez-Hackert and Stock, 1997a), whereas that encoded by *toxR*cyt-2 has the entire 170-amino-acid cytoplasmic domain of ToxR up to but not including the transmembrane domain of the wild-type protein. *toxR*mem encodes the cytoplasmic domain, the transmembrane domain and two predicted periplasmic amino acids. *toxR*peri encodes a protein with roughly one-third of the wild-type periplasmic sequences. We confirmed the predicted localization properties of each truncated protein by preparing subcellular fractions from a *toxR* deletion mutant of classical *V. cholerae* strain O395 (EK307) expressing these truncated proteins and subjecting these to immunoblot analysis with antibodies directed against ToxR (Fig. 2). ToxRcyt-1 and ToxRcyt-2, which lack the transmembrane domain of ToxR, fractionated completely with the cytoplasm. Full-length ToxRmem and ToxRperi, which contain the transmembrane domain of ToxR, were detected in both cytoplasmic and membrane fractions, and putative degradative products of each protein were also detected. Like wild-type ToxR, full-length ToxRmem and ToxRperi were not removed from the membrane fraction with 1 M NaCl, suggesting that they are integral inner membrane proteins and are not peripherally associated with the inner membrane through ionic interactions with other factors. Wild-type ToxR and full-length ToxRmem and ToxRperi detected in the cytoplasm may result from residual membrane contamination of the cytoplasmic fraction, may represent newly synthesized protein that has not localized to the inner membrane or, in the case of the last two, may be the result of improper localization resulting from lack of periplasmic sequences that might play a role in normal localization of ToxR. To investigate the domain requirements of ToxR for regulatory activity, *toxR*cyt-1, *toxR*cyt-2, *toxR*mem and *toxR*peri were tested for their ability to complement the *toxR* deletion mutant EK307, and dependence on TcpP was determined by assaying their function in strain EK459, a *toxR tcpP* double mutant (see below).

The cytoplasmic domain of ToxR is sufficient for regulation of ompU *and* ompT *expression, but not for* toxT *activation*

Analysis of total proteins by SDS-PAGE from both wild-type and *toxR* mutant *V. cholerae* (EK307) complemented
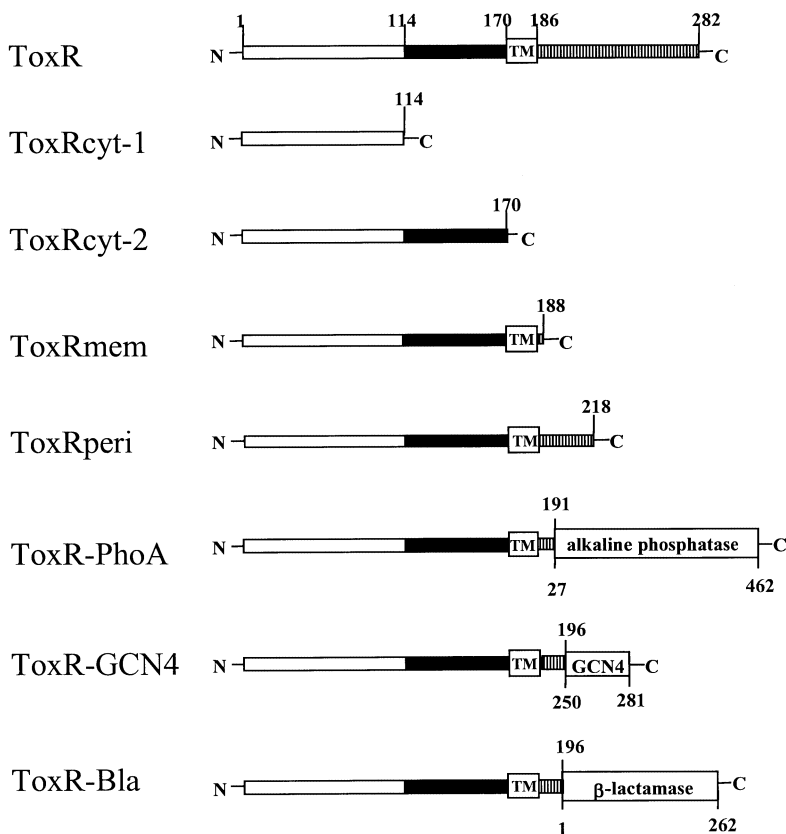

Fig. 1. Representation of the ToxR truncations and fusions used in this study. All proteins are depicted with the N-terminus on the left and the C-terminus on the right. Amino acid numbering is based upon N-terminal sequence analysis of ToxR performed by Pfau and Taylor (1998). Numbers above each protein refer to amino acids of ToxR, whereas numbers below refer to amino acids of each heterologous fusion domain. The open rectangle that initiates at the N-terminus represents the cytoplasmic, OmpR-homologous domain of ToxR, which ends at residue 114. The filled rectangle represents the remainder of the cytoplasmic domain, which ends at residue 170; the transmembrane domain is labelled TM and encompasses amino acids 171–186; the ToxR periplasmic domain is represented by the striped rectangle and encompasses amino acids 187–282. The heterologous domain in each ToxR fusion protein is labelled.
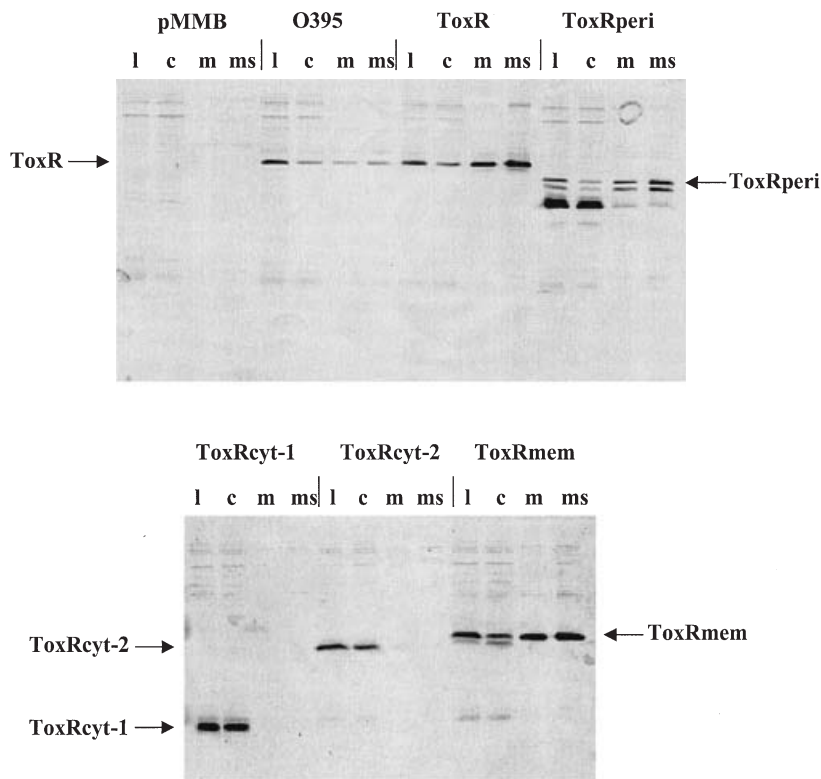
Fig. 2. Subcellular localization of the various ToxR truncates. Overnight cultures of O395 and the *toxR* mutant strain (EK307) containing pMMB66EH, pToxR, pToxRcyt-1, pToxRcyt-2, pToxRmem or pToxRperi were diluted 1:100 in LB plus 1 mM IPTG and grown at 30°C for 2 h. Cells were harvested and subjected to the fractionation protocol described in *Experimental procedures*. Equal percentages of total lysate (l), cytoplasm (c), membrane (m) and NaCl-washed membrane (ms) were analysed by immunoblotting with ToxR antisera as described in *Experimental procedures*. β-Galactosidase (Miller, 1972) and NADH oxidase assays (Osborn *et al.*, 1972) were performed to determine the efficiency of fractionation. Membrane fractions contained 70–85% of the total NADH oxidase activity, and cytoplasmic fractions contained 90–100% of the total β-galactosidase activity.

with a plasmid expressing wild-type ToxR showed the outer membrane protein expression profile characteristic of wild-type *V. cholerae*: high-level expression of OmpU and minimum expression of OmpT (Fig. 3, lanes 1 and 4). As expected, deletion of *toxR* resulted in loss of OmpU and derepression of OmpT (Fig. 3, lane 3). In contrast, Omp expression in a *tcpP* deletion strain was identical to that observed in the wild-type background (Fig. 3, lane 2),
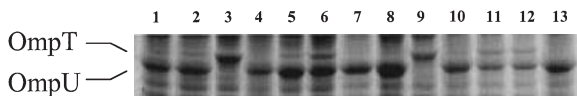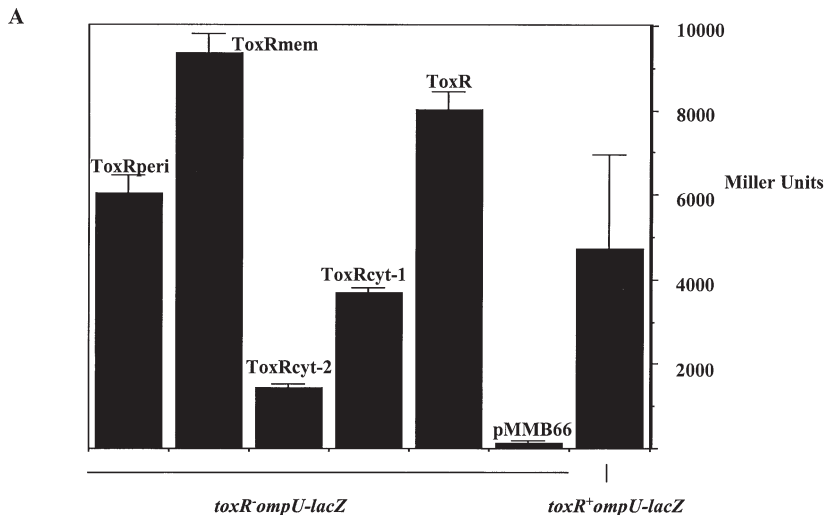

Fig. 3. SDS-PAGE analysis of OmpU and OmpT expression. Overnight cultures of strains carrying ToxR derivatives in pMMB66HE (lanes 4–8) were diluted 1:100 in LB plus 1 mM IPTG and grown at 30°C for 8 h. Strains carrying ToxR derivatives in pBR322 (lanes 10–13) were also grown at 30°C for 8 h. Whole-cell lysates were prepared, and OD$_{600}$ equivalents of protein were subjected to SDS-PAGE, followed by Coomassie brilliant blue staining. The positions of OmpU and OmpT are indicated and easily seen in lane 1 (O395) and lane 3 (EK307/pMM66HE) respectively. Lane 2 shows the outer membrane profile of strain RY1, which is a Δ*tcpP* derivative of O395. Lanes 4–13 are proteins from EK307 transformed with (lane designation): pToxR (4), pToxRcyt-1 (5), pToxRcyt-2 (6), pToxRmem (7), pToxRperi (8), pBR322 (9), pToxR (10), pToxR-PhoA (11), pToxR-GCN4 (12) and pToxR-Bla (13).

demonstrating that regulation of OmpU and OmpT is independent of TcpP, as predicted by the previous observation that Omp regulation is also independent of ToxT (Champion *et al.*, 1997). When complemented with a plasmid expressing ToxRcyt-1, the *toxR* mutant strain EK307 exhibited activation of OmpU with concomitant repression of OmpT (Fig. 3, lane 5). Activation of *ompU* transcription by ToxRcyt-1 was quantified by assaying β-galactosidase expression in a *toxR* mutant with a chromosomal *ompU–lacZ* gene fusion (EK410). By this measure, ToxRcyt-1 activated *ompU* transcription 30-fold compared with the mutant strain harbouring only the cloning vector (Fig. 4A). Repression of *ompT* transcription was measured directly by a primer extension assay using a radiolabelled *ompT* primer (Li *et al.*, 2000) and RNA from the *V. cholerae toxR* mutant strain EK307 expressing ToxRcyt-1. The level of *ompT* mRNA in the *toxR* mutant strain expressing ToxRcyt-1 was greatly reduced compared with the *toxR* mutant strain containing only the cloning vector pMMB66EH (Fig. 4B). ToxRcyt-1 was capable of repressing *ompT* transcription to the same extent as that observed with full-length ToxR expressed from a plasmid. We take these results to mean that the ToxR winged-helix domain alone is capable of regulating outer membrane protein production and, therefore, that DNA binding and transcriptional regulation by ToxR can occur in the absence of its mem-
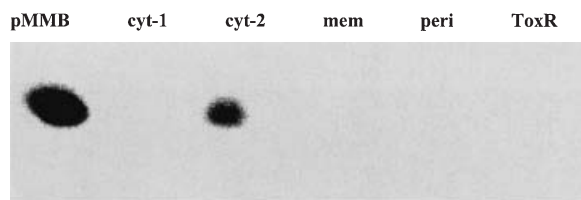
A

B

brane localization in *V. cholerae*. Although membrane localization is not required for transcription activation by ToxR, it may slightly enhance the ability of ToxR to activate the *ompU* promoter, based on the approximately twofold reduction in *ompU* promoter activation relative to plasmid-encoded full-length ToxR (Fig. 4A).

Unlike *omp* gene regulation, maximal *toxT* expression requires both ToxR and TcpP (Häse and Mekalanos, 1998; Murley *et al.*, 1999; Krukonis *et al.*, 2000). The observation that membrane localization is apparently not an absolute requirement for ToxR to regulate *omp* gene expression led us to investigate whether it is a necessary parameter for activation of *toxT* transcription. This was done by a primer extension assay using a radiolabelled *toxT* primer and RNA from *toxR* or *toxR tcpP* mutants of *V. cholerae* expressing ToxRcyt-1 (Yu and DiRita, 1999). We tested both strain backgrounds to ensure that any ToxR activity detected with ToxRcyt-1 on the *toxT* promoter retained wild-type dependence on TcpP.

Primer extension performed on RNA prepared from wild-type strain O395 after 1:100 dilution of an overnight culture revealed a *toxT* primer extension product within

2 h after the dilution (Fig. 5A), corresponding to mRNA initiating at the *toxT* promoter as described previously with this strain (Yu and DiRita, 1999). The intensity of this product decreased over the course of the experiment (data not shown), again in keeping with previous observations regarding *toxT* regulation in O395 (Yu and DiRita, 1999). A similar pattern of *toxT* transcription was observed when full-length ToxR was expressed from a plasmid in *toxR* mutant *V. cholerae* (Fig. 5A), indicating that plasmid-expressed ToxR complements the *toxR* mutation for *toxT* transcription. Activation of *toxT* was dependent on TcpP, as no message was detectable in RNA from the *toxR tcpP* double mutant (Fig. 5A).

ToxRcyt-1 was unable to activate expression of *toxT* despite its ability to activate *ompU* transcription to near wild-type levels (Fig. 5A). Although a small quantity of *toxT* primer extension product was observed at 2 h after dilution in the *toxR* mutant strain expressing ToxRcyt-1, this amount is not above the background level of transcription observed in the *toxR* mutant harbouring only the cloning vector. This small amount of transcription is probably the result of TcpP activity, given that it is not observed
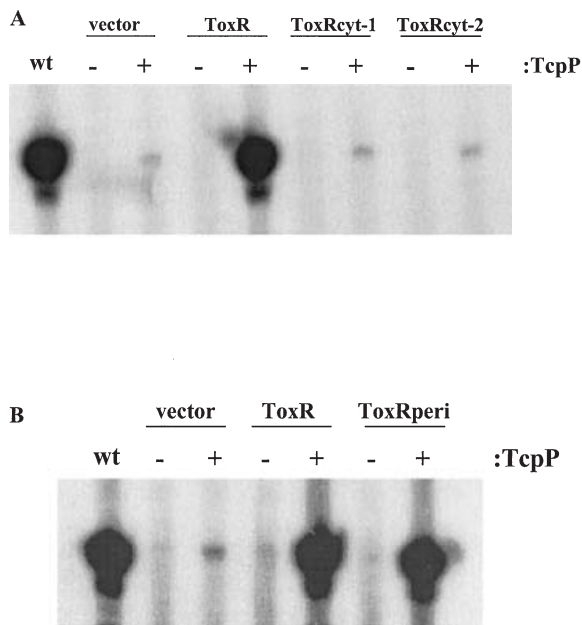
by primer extension of RNA isolated from a strain lacking both TcpP and ToxR (Fig. 5A, *tcpP*⁻ lanes). Analysis of TcpA production by immunoblotting and CT production by GM1-ELISA analysis were consistent with the *toxT* transcription data shown in Fig. 5A. Compared with cultures expressing wild-type ToxR, those expressing ToxRcyt-1 produced undetectable levels of TcpA by immunoblotting (data not shown) and were reduced for CT production by over 1000-fold (253 versus 0.24 ng of CT ml^{-1} supernatant/OD$_{600}$). Modulation of ToxRcyt-1 levels in EK307 by varying IPTG concentrations had no effect on the inability of this protein to activate *toxT* transcription (data not shown).

The other cytoplasmic form of ToxR, ToxRcyt-2, was similarly unable to activate *toxT* transcription to high levels when tested in both *toxR* and *toxR tcpP* double mutants strains (Fig. 5A). As with ToxRcyt-1, analysis of TcpA and CT expression also led us to conclude that ToxRcyt-2 is incapable of activating *toxT* gene expression to any relevant levels (data not shown) However, like ToxRcyt-1, ToxRcyt-2 activated *ompU* expression, which was observed by both SDS-PAGE (Fig. 3) and β-galactosidase activity expressed from an *ompU–lacZ* fusion in *V. cholerae* (Fig. 4A). ToxRcyt-2 activated *ompU–lacZ* only 11-fold over background, compared with activation levels of 60-fold for full-length ToxR and 30-fold for ToxRcyt-1. Additionally, ToxRcyt-2 repressed *ompT* transcription, as measured by primer extension, although not to the same level as ToxRcyt-1 (Fig. 4B). These results confirm our observation that the cytoplasmic domain of ToxR alone is insufficient to activate *toxT* in conjunction with TcpP, yet can

still regulate the TcpP-independent *ompU* and *ompT* promoters. The difference in *ompU* activation and *ompT* repression between ToxRcyt-1 and ToxRcyt-2 suggests that the cytoplasmic region of ToxR between the OmpR-homologous winged-helix domain and the transmembrane domain may have a slight inhibitory effect on the ability of the DNA-binding domain to function in the absence of membrane localization (see *Discussion*).

Functional analysis of the ToxR periplasmic domain

The results presented in the preceding sections demonstrate that cytoplasmic localization of the DNA-binding domain of ToxR results in lack of detectable *toxT* transcription and suggest that the transmembrane domain, the periplasmic domain or both are required for this process. To address the role of the periplasmic domain in TcpP-dependent *toxT* promoter activation, we analysed alleles of *toxR* in which the periplasmic domain was modified either by truncation or by being replaced with non-ToxR sequences.

An allele encoding a protein lacking two-thirds of the periplasmic domain (*toxR*peri) was cloned into the expression vector pMMB66EH. As with the other constructs in this study, ToxRperi was tested in *toxR* and *toxR tcpP* mutant strains of *V. cholerae* for its ability to activate *toxT* transcription. In *toxR* mutant *V. cholerae* strain EK307, we observed *toxT* activation by ToxRperi (Fig. 5B). In fact, this form of ToxR could activate *toxT* transcription with greater efficiency than wild type, judging from the level of *toxT* primer extension product from RNA of the *toxR* mutant

complemented with pToxRperi compared with that from a strain expressing wild-type ToxR at time points beyond the 2 h point shown in Fig. 5B (data not shown). Activation of *toxT* by ToxRperi retained wild-type dependence on TcpP, as transcription was undetectable in the *toxR tcpP* double mutant strain EK459 (Fig. 5B). Expression of *toxT* by ToxRperi was sufficient for subsequent expression of both TcpA (data not shown) and CT; in the case of CT, the *toxR* mutant strain EK307 expressing ToxRperi expressed 1033 ng of CT ml^{-1} supernatant/OD$_{600}$, which was roughly five times more than produced in EK307 expressing ToxR. These results suggest that membrane-bound ToxRperi is sufficient to activate the *toxT* promoter, and that a majority of the ToxR periplasmic domain is not required for this event. An alternative interpretation is that the cytoplasmic degradation products of ToxRperi observed in Fig. 2 activate *toxT* transcription, but we do not favour this interpretation because two different cytoplasmic forms of ToxR (ToxRcyt-1 and ToxRcyt-2) do not themselves activate the *toxT* promoter.

The periplasmic truncation had no effect on Omp regulation, because ToxRperi also regulated OmpU and OmpT expression like wild type (Fig. 3, lane 8). Activation of *ompU–lacZ* with ToxRperi was 48-fold, nearly the same level as that observed with wild-type ToxR (Fig. 4A), and ToxRperi was capable of repressing *ompT* transcription to the same level as full-length ToxR, as measured by primer extension (Fig. 4B). The cytoplasmic degradation products of ToxRperi may contribute to the regulation of OmpU and OmpT expression, as ToxRcyt-1 and ToxRcyt-2 have this capability.

Analysis of TcpP dependence of ToxR fusion proteins

Other periplasmically altered ToxR derivatives that we investigated for their ability to co-operate with TcpP for *toxT* activation were those in which the periplasmic domain was replaced by non-ToxR sequences. A number of observations have been reported using fusions proteins in which the periplasmic domain of ToxR is replaced by proteins such as alkaline phosphatase, the leucine zipper domain from the yeast regulatory protein GCN4 and periplasmic β-lactamase. These fusion proteins were constructed with the aim of determining whether or not predicted alterations in the multimeric structure of ToxR caused by these domains have an effect on ToxR function (Ottemann and Mekalanos, 1995). Observations with these fusion proteins, made by different groups, have not been consistent with one another (see *Discussion*) and, in any event, previous experiments with them were not designed specifically to address the role of TcpP in *toxT* transcription (Ottemann and Mekalanos, 1995). We therefore analysed transcription activation of *toxT* by TcpP in the presence of these fusion proteins.

ToxR fusion derivatives were tested with three different periplasmic domains in place of the natural ToxR periplasmic domain. These were fusions to alkaline phosphatase (PhoA) (DiRita and Mekalanos, 1991), which functions as a dimer in the periplasm (Wanner, 1996), GCN4, a leucine zipper dimerization domain (Hu *et al.*, 1990; Ottemann and Mekalanos, 1995) and β-lactamase (Bla) (Ottemann and Mekalanos, 1995), which functions as a monomer in the periplasm (Schlam *et al.*, 1989; Oefner *et al.*, 1990; Herzberg, 1991). Each fusion protein contains the cytoplasmic and transmembrane domains of ToxR, five (ToxR–PhoA) or 10 (ToxR–Bla and ToxR–GCN4) periplasmic amino acids from ToxR and the particular heterologous domain (Fig. 1). All are predicted to localize to the inner membrane by virtue of the ToxR transmembrane domain (ToxR–PhoA has previously been demonstrated experimentally to localize to this compartment; Miller *et al.*, 1987). Plasmids encoding each were mobilized into *toxR* mutant and *toxR tcpP* double mutant *V. cholerae* strains and, as with other ToxR derivatives used in this study, primer extension of *toxT* mRNA over time after 1:100 dilution of an overnight culture was used to determine the ability of the fusions to activate *toxT* transcription. In addition to analysing mRNA expression, we analysed outer membrane protein expression by SDS-PAGE.

Each fusion protein activated *toxT* transcription to levels equivalent to that observed with wild-type ToxR, and the activity of each was strictly dependent on TcpP (Fig. 6). In addition, activation of *toxT* transcription by each ToxR fusion protein led to the expression of high levels of TcpA and CT, as determined by immunoblotting with TcpA antisera and by GM1-ELISA (data not shown). Finally, each
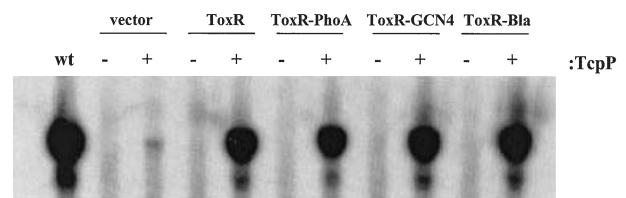
Fig. 6. *toxT* primer extension analysis in cells expressing ToxR fusion proteins. Overnight cultures were diluted 1:100 in LB and grown at 30°C. Aliquots of each culture were removed at 2, 4, 6 and 8 h after dilution, and RNA was prepared. Equal amounts of RNA were subjected to primer extension using a radiolabelled *toxT* primer as described in *Experimental procedures*. The 2 h time point is shown. The various ToxR fusions were analysed in a *toxR tcpP* double deletion strain of O395 (EK459, represented by a minus sign, indicating lack of TcpP expression), and in a *toxR* deletion strain of O395 in which *tcpP* is wild type (EK307, represented by a plus sign, indicating TcpP expression). Both strains contain the expression vector pBR322, pVM16 (expressing wild-type ToxR), pToxRPhoA (expressing ToxR–PhoA), pKO9 (expressing ToxR–GCN4) or pKO21 (expressing ToxR–Bla). The lane labelled wt represents results obtained with strain O395.

ToxR fusion protein regulated Omp expression like wild type, leading to the production of OmpU and repression of OmpT (Fig. 3), consistent with high-level expression of *ompU–lacZ* that we observed by expressing these proteins in *V. cholerae* strain EK410 (data not shown).

These data demonstrate that the fusion proteins behave like wild-type ToxR with respect to their dependence on TcpP for *toxT* activation and subsequent downstream expression of *tcpA* and *ctxAB*, even though they consist mainly of non-ToxR sequence in the periplasm. Additionally, the different predicted structures of their periplasmic domains suggest that there is not a predictable dimeric or multimeric state that the ToxR periplasmic domain adopts to activate the *toxT* promoter.

Membrane localization of the amino-terminal winged-helix domain of ToxR is sufficient for toxT promoter activation

Analysis of ToxRperi and the ToxR fusion proteins demonstrated that neither the amino acid sequence nor the structure of the ToxR periplasmic domain is critical for *toxT* promoter activation by TcpP, which suggests that membrane localization of the winged-helix domain of ToxR is the critical parameter that must be satisfied for ToxR to contribute to activation of *toxT* transcription. To test this hypothesis, we constructed an allele encoding the ToxR cytoplasmic and transmembrane domains, plus two predicted periplasmic amino acids (*toxR*mem), and cloned this into the expression vector pMMB66EH (Fig. 1). ToxRmem was tested for its ability to activate *toxT* transcription in a TcpP-dependent manner by performing primer extension analysis as was done with the other ToxR derivatives.

Alone among the ToxR variants that we tested, the ability of ToxRmem to restore *toxT* transcription and lead to subsequent activation of *ctxAB* and *tcpA* in *toxR* mutant *V. cholerae* was dependent on its level of expression (Fig. 7A). When *toxR*mem expression was induced with 1 mM IPTG, *toxT* transcription could be activated, but it was not sustained over the time course of the experiment compared with the activation seen using full-length ToxR (Fig. 7A). By 4 h, the *toxT* primer extension product from cells expressing ToxRmem after induction with 1.0 mM IPTG was quite diminished compared with that from cells expressing full-length ToxR and was undetectable by 6 h (Fig. 7A; data not shown). This level of *toxT* transcription was insufficient to lead to the expression of genes downstream of *toxT* in the regulatory cascade, which was reflected in low levels of CT and TcpA detected at each time point. However, when 100-fold less IPTG was used to induce *toxR*mem, activation of *toxT* was restored to wild-type levels at the later time points (Fig. 7) and, in turn, expression of CT and TcpA was like wild type (Fig. 7B). At either concentration of IPTG, the observed activation
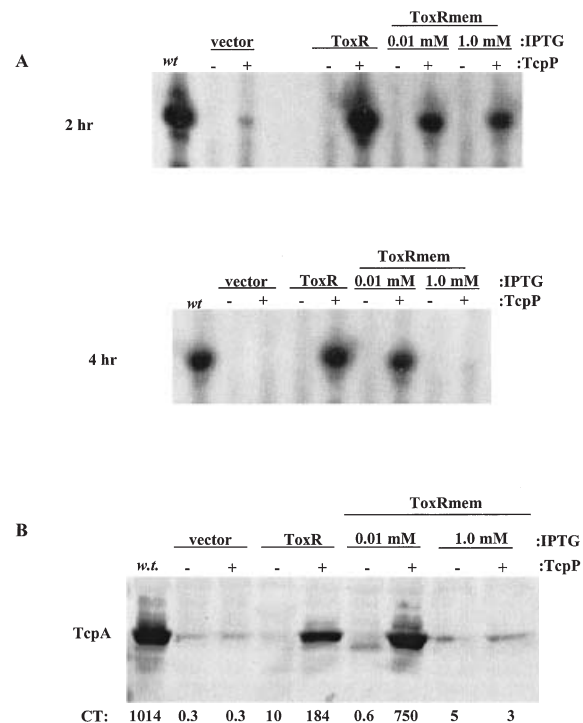

Fig. 7. Analysis of ToxRmem.
A. *toxT* primer extension analysis with ToxRmem. *Vibrio cholerae* cultures grown as for the primer extension experiment shown in Fig. 5 were used to prepare RNA for primer extension as described in *Experimental procedures*. IPTG (1 mM) was used, and the 2 h time point is shown. Plasmids encoding ToxR, ToxRmem or the cloning vector (pMMB66EH) alone were introduced into strains EK459 (*toxR⁻ tcpP⁻*; represented by a minus sign to indicate lack of TcpP expression) and EK307 (*toxR⁻ tcpP⁺* represented by a plus sign to indicate TcpP expression). The lower blot shows *toxT* primer extension analysis with ToxRmem expression induced with both 1 mM and 0.01 mM IPTG as indicated. Both 2 h and 4 h time points are shown to show the lack of sustained expression of *toxT* at the higher concentration of inducer. The lane labelled wt in both blots represents primer extension analysis of strain O395.
B. TcpA and CT expression. Overnight cultures were diluted 1:100 in LB plus either 1 mM IPTG or 0.01 mM IPTG as indicated and grown at 30°C. An aliquot of each culture was removed after overnight growth, and whole-cell lysates were prepared. OD$_{600}$ equivalents of whole-cell lysates were subjected to TcpA immunoblotting as described in *Experimental procedures*. The CT values, shown below the immunoblot, were derived from GM1-ELISA of equal volumes of supernatants from each overnight culture, as described in *Experimental procedures*. O395 is wild-type *V. cholerae.* The activity of ToxRmem was analysed in EK459 (*toxR⁻ tcpP⁻*, represented by a minus sign to indicate lack of TcpP expression) and EK307 (*toxR⁻ tcpP⁺*, represented by a plus sign to indicate TcpP).

of *toxT* transcription was strictly TcpP dependent. We take this apparent concentration dependence of ToxRmem, which was not observed with other variants analysed in this study (data not shown), to mean that, although ToxRmem can activate *toxT* transcription, it is detrimental to activation when expressed at high levels. Also, we conclude that a threshold level of *toxT* expression must be achieved for subsequent gene expression in the cas-

cade, given that detectable but unsustained activation of *toxT* by ToxRmem did not lead to virulence gene expression. These observations suggest that membrane-localized ToxRmem is sufficient to activate expression of *toxT*. Again, we argue against the possibility that the cytoplasmic degradation product of ToxRmem is responsible for activating *toxT* transcription because two different cytoplasmic forms of ToxR of similar size to this product (ToxR-cyt-1 and ToxRcyt-2) do not activate the *toxT* promoter.

As might be predicted given the results with ToxRcyt-1, ToxRcyt-2 and ToxRperi, *ompU* activation by ToxRmem in *V. cholerae* was similar to activation by ToxR, measured by both SDS-PAGE and analysis of β-galactosidase production from *ompU–lacZ*. In addition, levels of OmpT protein on SDS-PAGE and *ompT* mRNA measured by primer extension were repressed to levels observed with full-length ToxR (Figs 3 and 4).

Discussion

In this study, we performed a structure–function analysis of ToxR to understand better the relationship between the domain structure of ToxR and its regulatory function in *V. cholerae*. The simplest conclusion to draw from our data is that membrane localization of the ToxR winged-helix domain is required for ToxR and TcpP, which itself is membrane localized, to co-operate to activate the *toxT* promoter, but membrane localization of ToxR is not as critical for TcpP-independent regulation of the *ompU* and *ompT* promoters. This conclusion is based in part on our observation that the amino-terminal winged-helix domain alone (ToxRcyt-1), which fractionates completely in the cytoplasm, was unable to activate *toxT* expression but was capable of controlling *ompU* and *ompT* expression. This finding suggests that, for the *ompU* and *ompT* promoters, membrane localization of ToxR is not a prerequisite for DNA binding or interaction with RNA polymerase. ToxR-cyt-1 is also capable of binding the *toxT* promoter, as judged by its ability to repress basal *toxT–lacZ* activity in *E. coli* (data not shown), demonstrated previously to be a feature of DNA binding to this promoter by the wild-type protein (Higgins and DiRita, 1994). Biochemical experiments also demonstrated that purified ToxRcyt-1 binds the *toxT* promoter (J. A. Crawford and V. J. DiRita, unpublished). Therefore, the ability of ToxRcyt-1 to interact with the *ompU*, *ompT* and *toxT* promoter DNA suggests that the winged helix of ToxR, in the absence of membrane localization, adopts the necessary conformation for DNA binding.

Other groups have examined the ability of various cytoplasmic forms of ToxR to activate the *ctxAB* promoter in *E. coli*, which is a capability of wild-type ToxR. These groups observed that cytoplasmic ToxR alone did not activate the *ctxAB* promoter in *E. coli* (Kolmar *et al.*, 1995;

Pfau and Taylor, 1998; Dziejman *et al.*, 1999). However, when fused to a dimerization domain such as the leucine zipper domain of GCN4, cytoplasmic ToxR was capable of activating the *ctxAB* promoter (Kolmar *et al.*, 1995; Ottemann and Mekalanos, 1995; Dziejman *et al.*, 1999), although the magnitude of activation varied from group to group. These results suggest that the ability of cytoplasmic ToxR to activate the *ctxAB* promoter depends on the dimerization status of the ToxR DNA-binding domain in the cytoplasm. Analysis of *ompU* and *ompT* promoter regulation in this report suggests that the DNA-binding domain of ToxR alone, in the absence of an exogenous dimerization domain, functions well for regulation of *omp* gene expression. We conclude that facilitated dimerization of the ToxR winged-helix domain is not required for it to activate and repress the *ompU* and *ompT* promoters, although, based on the work of others described above, it may be required for ToxR to activate the *ctxAB* promoter. In support of this, when assayed in *E. coli*, ToxRcyt-1 did not activate a *ctx–lacZ* promoter fusion, but did activate an *ompU–lacZ* fusion 30-fold (data not shown). Collectively, these observations made by us and other groups suggest that mechanistic differences may exist in ToxR-mediated activation of the *ctxAB* and *ompU* promoters.

Although the cytoplasmic forms of ToxR used in this study are capable of controlling *ompU* and *ompT* gene expression in *V. cholerae*, we note that, when measured quantitatively, ToxRcyt-1 and ToxRcyt-2 were at least two-fold reduced in their ability to activate the *ompU* promoter (Fig. 4A); ToxRcyt-2 was also unable to repress *ompT* fully (Fig. 4B). This suggests that, although membrane localization is not an absolute requirement for ToxR to interact with the *ompU* and *ompT* promoters, it may enhance the activity of ToxR. One of two mechanisms may explain the reduced activity of ToxRcyt-2: first, the expression level of ToxRcyt-2 appears to be slightly reduced compared with ToxRcyt-1 (Fig. 2); secondly, residues 115–170 of ToxR-cyt-2 may have an inhibitory effect on the ToxR DNA-binding/transcription activation domain (residues 1–114). This potential inhibitory effect of residues 115–170 may partially explain observations made by Ottemann and Mekalanos (1995), who did not detect regulation of OmpU and OmpT in *V. cholerae* by a soluble form of ToxR consisting of the winged-helix domain plus the inhibitory segment from residues 115–170 fused to the leucine zipper of GCN4. In addition to the inhibitory effect of residues 115–170, the lack of OmpU and OmpT regulation observed by Ottemann and Mekalanos (1995) may be related to the fact that the ToxR–GCN4 hybrid was expressed in single copy from the *V. cholerae* chromosome. To observe regulatory activity, ToxR–GCN4 may need to be expressed in multicopy similar to ToxRcyt-2 in this study. How the other forms of ToxR used in this study, particularly ToxRcyt-1 and ToxRmem, would behave in *V.*

cholerae when expressed at physiological levels from the chromosome is a question that we are currently pursuing. The construction of such strains will also allow us to study the various forms of ToxR in an animal model, which will be important to investigate the apparent differences in control of the ToxR regulon under *in vivo* conditions versus *in vitro* growth (Lee *et al.*, 1999; 2001).

In all cases that we examined, TcpP was also required for activation of *toxT* transcription, ruling out the possibility that expression of a truncated form of ToxR may result in bypassing the requirement for TcpP in this system. The strict requirements of localizing the ToxR winged-helix domain to the membrane and the presence of TcpP for transcription of *toxT* to occur suggest that ToxR and TcpP must be in the same cellular compartment in order to function together to activate the *toxT* promoter (Fig. 8), consistent with conclusions reached by others (Ottemann

and Mekalanos, 1995; Dziejman *et al.*, 1999). This raises the question of whether or not cytoplasmic co-localization of ToxR and TcpP, or their DNA-binding/activation domains alone, would lead to *toxT* transcription. This aspect of the model is currently under investigation. We consider it unlikely that the observations made in this report concerning the activity of the *toxT* promoter can be explained by an effect on TcpP expression resulting from truncating ToxR, as it has been demonstrated that ToxR does not influence *tcpP* transcription (Carroll *et al.*, 1997).

The results presented in this study are consistent with a model based on recent work from our laboratory describing how ToxR and TcpP may interact at the *toxT* promoter to activate its transcription (Krukonis *et al.*, 2000). The TcpP binding site in the *toxT* promoter is between −51 and −32 (relative to the *toxT* transcription initiation site), downstream of the ToxR binding site
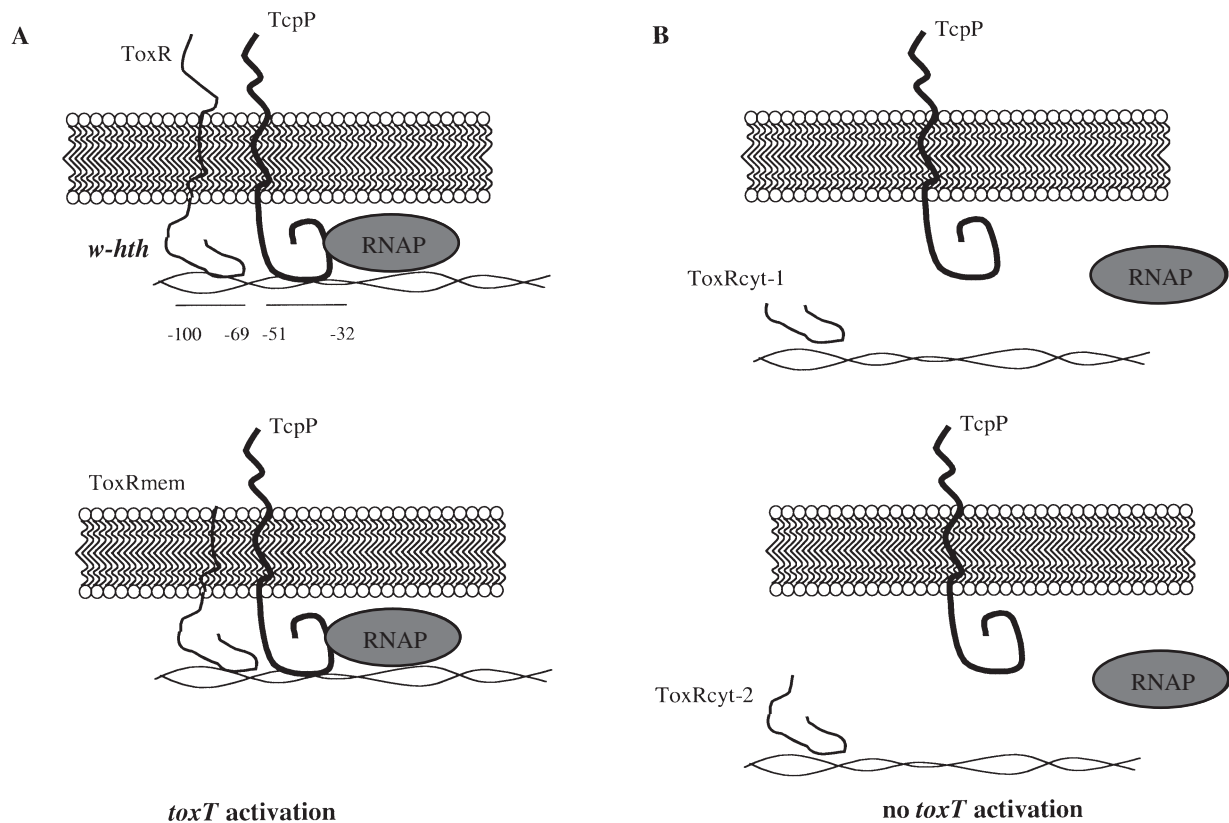
Fig. 8. Model for membrane co-localization of ToxR and TcpP required for *toxT* activation.
A. Two representative conditions that allow for transcription initiation of *toxT*. The top part depicts wild-type binding of ToxR and TcpP to their respective sites in the *toxT* promoter as demonstrated by Krukonis *et al.* (2000). The cytoplasmic DNA-binding domain of ToxR is a member of the winged-helix family of transcription activators. The lower part depicts activation of *toxT* transcription by ToxRmem, which has only the ToxR winged-helix transmembrane domain, as demonstrated in this report. Similar activation is also seen with other forms of ToxR provided that they localize to the membrane with TcpP.
B. Two cytoplasmically localized forms of ToxR, ToxRcyt-1 and ToxRcyt-2, which express functionally active ToxR DNA-binding domains, as evidenced by their ability to activate transcription of the TcpP-independent promoter of the *ompU* gene, but fail to support TcpP-mediated activation of *toxT*. In the model, DNA binding by membrane-localized ToxR may bring the *toxT* promoter to TcpP in order for it to bind DNA and stimulate RNA polymerase (RNAP) to activate transcription. Binding by ToxR is also known to clear the transcription repressor H-NS from the *toxT* promoter, thereby allowing transcription to ensue, as shown recently by Nye *et al.* (2000).

between −100 and −69. Krukonis *et al.* (2000) proposed that ToxR may contribute to *toxT* activation by recruiting the promoter to the membrane, where TcpP resides, or by stabilizing a weak association between TcpP and the promoter, thereby allowing it to interact with polymerase and activate transcription. The stabilization model is supported by the observation that membranes containing TcpP alone are capable of binding *toxT* promoter DNA, even to the extent of providing protection against DNase I digestion, but the concentration of TcpP-containing membranes required for binding DNA is 10-fold higher than that required for binding by ToxR/TcpP-containing membranes (Krukonis *et al.*, 2000). Our demonstration in this report that the ToxR DNA-binding domain must be in the membrane in order for membrane-localized TcpP to activate *toxT* transcription suggests that the two proteins may form an activating complex in the membrane with ToxR upstream of TcpP and TcpP contacting RNA polymerase (Fig. 8). How RNA polymerase is brought to this membrane complex remains to be determined, although association of RNA polymerase with the bacterial cytoplasmic membrane through interaction with membrane regulatory factors has been reported by others (Rowen and Deretic, 2000). Another role for ToxR in activating *toxT*, recently demonstrated by Nye *et al.* (2000), is to counteract binding by H-NS, binding of which near the ToxR binding site in the *toxT* promoter represses transcription; this antirepressor function of ToxR is not precluded by our model for co-operation between ToxR and TcpP in *toxT* activation (Fig. 8).

Periplasmic truncations appear to affect the stability of ToxR, based on our observation that both ToxRmem and ToxRperi are partially degraded to cytoplasmic species in *V. cholerae*, similar to what has been observed previously with different forms of ToxR expressed in *E. coli* and *Salmonella typhimurium* (DiRita and Mekalanos, 1991; Pfau and Taylor, 1998). Although it is possible that these cytoplasmic degradation products of each protein are responsible for the *toxT* promoter activation that we observe, we do not consider this a likely possibility. We favour instead the interpretation that the membrane-bound, full-length form of each protein activates the *toxT* promoter, because two *bona fide* cytoplasmic forms, ToxRcyt-1 and ToxRcyt-2, did not activate *toxT* transcription. Considering that the periplasmic domain of ToxR can be so grossly altered and even removed with little effect on *toxT* transcription activation, any functional interaction that may occur between TcpP and ToxR probably does not take place in the periplasm in the way that interaction between ToxR and ToxS is purported to (DiRita and Mekalanos, 1991). In this context, it is difficult to reconcile how ToxR appears to activate gene expression so well lacking a periplasmic domain that purportedly interacts with ToxS, and to do so requires more experimentation. Furthermore, replace-

ment of the ToxR transmembrane domain with heterologous sequences does not affect the ability of ToxR to function in *V. cholerae*, suggesting that ToxR and TcpP do not interact through their transmembrane domains (Ottemann and Mekalanos, 1995).

We have presented evidence suggesting that membrane localization of the winged HTH domain of ToxR is critical for activation of *toxT* and subsequent regulatory events leading to CT and TCP production, but that this function is not as critical for regulating *ompU* and *ompT*. These findings imply that membrane localization is not an absolute requirement for DNA binding and transcription activation by ToxR but, instead, is required specifically for it to activate the *toxT* promoter in conjunction with TcpP through a mechanism that is yet to be elucidated.

Experimental procedures

Bacterial strains and plasmids

The *E. coli* and *V. cholerae* strains used in this study are listed in Table 1 and were maintained at −70°C in LB medium plus 20% glycerol. Strains were grown in LB medium at 30°C. Antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹; streptomycin, 100 µg ml⁻¹; and kanamycin, 30 µg ml⁻¹. Plasmids were introduced into *V. cholerae* strains through triparental mating with *E. coli* strain MM294 (pRK2013) as a donor of mobilization functions, and into *E. coli* by transformation.

DNA manipulations

Polymerase chain reaction (PCR) products containing various amounts of *toxR* were generated using either *Taq* DNA polymerase (Gibco BRL) or the Expand™ High Fidelity PCR system (Boehringer Mannheim) using the manufacturer's specified procedure. PCR templates were either plasmid VJ21 (Miller *et al.*, 1989) or chromosomal DNA from *V. cholerae* strain O395. Synthesized primers contained added recognition sequences for restriction endonucleases to facilitate directional cloning. PCR products were purified by agarose gel electrophoresis followed by gel extraction using the QIAEX II gel extraction system (Qiagen). Cloning procedures were carried out using standard protocols (Sambrook *et al.*, 1989). Double-stranded sequencing of plasmids was performed as reported previously (Higgins *et al.*, 1992).

Growth conditions

Time course experiments for each strain were performed as follows: overnight cultures grown in LB at 30°C were diluted 1:100 into fresh LB medium (and IPTG as necessary) and grown at 30°C. At 2, 4, 6 and 8 h after dilution, the OD_{600} of each culture was measured, and aliquots of each culture were collected by centrifugation for RNA isolation (to analyse *toxT* mRNA amounts) and for whole-cell lysate preparation (to analyse TcpA, OmpU and OmpT expression). An aliquot

Table 1. Strains and plasmids.

Strain/plasmid	Description	Reference/source
Strain		
V. cholerae		
O395	Str^r	Laboratory collection
EK307	O395 Δ*toxR*	Krukonis *et al.* (2000)
EK459	O395 Δ*toxR* Δ*tcpP*	Krukonis *et al.* (2000)
RY1	O395 Δ*tcpP*	Yu and DiRita (1999)
EK383	O395 [*lacZ::*p*ompU–lacZ*]	Laboratory collection
EK410	EK307 [*lacZ::*p*ompU–lacZ*]	Krukonis *et al.* (2000)
Plasmids		
pMMB66EH	Cloning vector; Ap^R	Morales *et al.* (1991)
pAlf (pToxR)	pMMB66EH with *toxR*	Gift from Dr J. Sanchez, Facultad de Medicina, UAEM
pToxRcyt-1	pMMB66EH with *toxR*cyt-1 insert	This work
pToxRcyt-2	pMMB66EH with *toxR*cyt-2 insert	This work
pToxRmem	pMMB66EH with *toxR*mem insert	This work
pToxRperi	pMMB66EH with *toxR*peri insert	This work
pBR322	Cloning vector; Ap^R, Tc^R	Bolivar *et al.* (1977)
pVM16	pBR322 tc::*toxR*	Miller *et al.* (1989)
pToxRPhoA-S	pBR322 with *toxR*′∠*phoA*	DiRita and Mekalanos (1991)
pKO9	pBR322 with *toxR*′∠GCN4	Ottemann and Mekalanos (1995)
pKO21	pBR3222 with *toxR*′∠*bla*	Ottemann and Mekalanos (1995)

of each culture supernatant was saved for analysis of CT expression. Whole-cell lysates were made by resuspending the cell pellet in 250 μl of LB plus 250 μl of 2× SDS-PAGE sample buffer and boiled for 5 min.

Primer extension

RNA was isolated from *V. cholerae* cultures using Trizol reagent (Gibco BRL) according to the manufacturer's recommended protocol. For *ompT* primer extension, RNA was isolated from mid-logarithmic cultures, while RNA was isolated from 2, 4, 6 and 8 h time course samples for *toxT* primer extension. The RNA samples were quantified by measuring A_{260} and electrophoresed on 1% agarose gels to check for degradation. Each RNA sample (20 μg) was used in a primer extension reaction, using the *toxT*-specific primer 5′-CATTAGTTTGAAAAGATTTTTTCCCAATCAT-3′, which initiates at +98 in *toxT* (Higgins and DiRita, 1994), or the *ompT*-specific primer 5′-GCACTGCGAGTGCTAATAGA-3′, which initiates at +140 in *ompT* (Li *et al.*, 2000). Each primer (10 pmol) was end-labelled using 30 μCi of [γ-^{32}P]-ATP (>3000 Ci mmol^{-1}; Amersham) and T4 polynucleotide kinase (NEB) as described previously (Sambrook *et al.*, 1989). Approximately 0.2 pmol of labelled primer was mixed with 20 μg of each RNA sample. An aliquot of 2 μl of hybridization buffer (0.5 M KCl, 0.25 M Tris-HCl, pH 8.3) and water to 10 μl were added. Reactions were incubated at 95°C for 1 min, transferred to 55°C for 2 min and placed on ice for 15 min. Samples of 5 μl of each annealing reaction were added to tubes containing 1 μl of a 2.5 mM dGTP, dATP, dTTP, and dCTP mix, 1 μl of 0.1 M dithiothreitol (DTT), 2 μl of reverse transcriptase buffer (250 mM Tris, pH 8.3, 375 mM KCl, 15 mM MgCl$_2$) and 1 μl (200 units) of Superscript II RNase H$^-$ reverse transcriptase (Gibco BRL). Reactions were incubated at 44°C for 45 min, and then 5 μl

of Sequenase stop buffer (United States Biochemical) was added to terminate the reactions. Reactions were heated at 95°C for 3 min and electrophoresed on 6% denaturing polyacrylamide gels.

TcpA immunoblotting

OD$_{600}$ equivalents of whole-cell lysates prepared in SDS-PAGE sample buffer were electrophoresed on a 15% polyacrylamide gel with a 5% stacking gel, transferred to nitrocellulose and blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline plus 0.1% Tween 20 (TBS-T) overnight at 4°C. Next, the blot was incubated 2–3 h at room temperature with TcpA polyclonal antisera (kindly supplied by R. Taylor, Dartmouth Medical School), diluted 1:100 000 in 5% milk–TBS-T. The blot was then washed 3 × 10 min in TBS-T and incubated for 1 h at room temperature with goat anti-rabbit IgG linked to alkaline phosphatase (Gibco BRL) diluted 1:1000 in 5% milk–TBS-T. The blot was then washed 3 × 10 min with TBS-T. The chromogenic substrates for alkaline phosphatase, nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP), both obtained from Gibco BRL, were added to develop the blot.

Enzyme-linked immunosorbent assay (ELISA)

An equal volume of supernatant from each time course culture was added to 96-well plates coated with GM1, the cholera toxin receptor. After a 1–2 h room temperature incubation, the plates were washed three times with phosphate-buffered-saline (PBS; pH 7.4)–0.2% BSA–0.05% Tween 20. CT antisera, specific for the B subunit (kindly supplied by M. Bagdasarian, Michigan State University), was diluted 1:1000 in PBS–0.2% BSA–0.05% Tween 20 and

added to each well. After a 1–2 h room temperature incubation, the plates were washed three times with PBS–0.2% BSA–0.05% Tween 20. Goat anti-rabbit antibodies linked to alkaline phosphatase (Gibco BRL) were diluted 1:1000 in PBS (pH 7.4)–0.2% BSA–0.05% Tween 20 and added to each well. After a 1–2 h room temperature incubation, the plates were washed three times with PBS (pH 7.4)–0.2% BSA–0.05% Tween 20, and *p*-nitrophenyl phosphate (Sigma) was added. Absorption at 420 nm was measured and converted to CT concentration by normalizing the A_{420} value to the absorption value generated by a known concentration of CT present on the 96-well plate. This value was divided by the OD_{600} of the culture to yield CT units reported as ng of CT ml^{-1} supernatant/OD_{600}.

SDS-PAGE

OmpU and OmpT expression was analysed by subjecting OD_{600} equivalents of 8 h whole-cell lysate samples to SDS-PAGE, followed by Coomassie brilliant blue staining.

Cell fractionation

The various *V. cholerae* strains subjected to the fractionation protocol were grown overnight at 30°C in LB medium, diluted 1:100 the following day in LB medium plus 1 mM IPTG and grown for 2 h at 30°C. Samples of 40 ml of each strain were pelleted by centrifugation and resuspended in 100 µl of 0.1 M Tris, pH 8.0. To this was added 200 µl of 0.1 M Tris (pH 8.0)–1 M sucrose, 20 µl of 10 mM EDTA, pH 8.0, and 20 µl of 10 mg ml^{-1} lysozyme (made fresh in water). Samples were allowed to digest on ice for 15 min. Next, 640 µl of water was added, and the samples were allowed to digest on ice for an additional 15 min. The samples were frozen in a dry ice–ethanol bath and allowed to thaw in cold water. A sample of 20 µl of DNase I (0.25 mg ml^{-1}, made fresh in water) was added. The samples were next sonicated (5 s pulse) and centrifuged at 5000 *g* for 2 min at 4°C to pellet unlysed cells. Each sample (500 µl) was then centrifuged at 15 000 *g* for 1 h at 4°C to pellet the inner and outer membranes. Membrane pellets were resuspended in 50 µl of 0.1 M Tris, pH 8.0, 100 µl of 0.1 M Tris, pH 8.0–1 M sucrose, 10 µl of 10 mM EDTA, pH 8.0, and 340 µl of water. Equal percentages of total lysate, cytoplasmic fraction and membrane fraction were analysed for the presence of the cytoplasmic marker β-galactosidase (Miller, 1972) and the inner membrane marker NADH oxidase (Osborn *et al.*, 1972). Membrane fractions contained 70–85% of the NADH oxidase activity, and cytoplasmic fractions contained 90–100% of the β-galactosidase activity. An aliquot of each membrane fraction was washed with 1 M NaCl, pelleted by centrifugation at 15 000 *g* for 1 h at 4°C and resuspended in water. Equal percentages of total lysate, cytoplasmic fraction, membrane fraction and NaCl-washed membrane fraction were mixed with SDS-PAGE sample buffer and boiled for 5 min. Equal percentages of each fraction were electrophoresed on a 15% polyacrylamide gel with a 5% stacking gel, transferred to nitrocellulose and probed with ToxR polyclonal antisera (kindly supplied by J. Mekalanos, Harvard Medical School) according to the protocol used for the TcpA immunoblots described above.

β-Galactosidase assays

β-Galactosidase assays were done according to the method of Miller (1972). Cultures of each *V. cholerae* strain were grown overnight in LB at 30°C. The following day, each culture was diluted 1:100 into fresh LB medium (and 1 mM IPTG as necessary) and grown at 30°C to mid-logarithmic phase, at which time the β-galactosidase activity was measured.

Acknowledgements

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