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Specific binding of the diphtheria *tox* regulatory element DtxR to the *tox* operator requires divalent heavy metal ions and a 9-base-pair interrupted palindromic sequence

(*tox* repressor/gene regulation/mobility shift assay)

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ABSTRACT **The structural gene for diphtheria toxin, *tox*, is carried by a family of closely related corynebacteriophages; however, the regulation of *tox* expression is controlled by a *Corynebacterium diphtheriae*-encoded regulatory element, *dtxR*. The molecular cloning and sequence analysis of *dtxR* was recently described. Previous studies have suggested that DtxR-mediated regulation of the diphtheria *tox* operator involves the formation of an iron–repressor complex, which specifically binds to the *tox* operator. We have expressed and purified DtxR from recombinant *Escherichia coli*. Immunoblot analysis shows DtxR to be a single M_r 28,000 protein band in both recombinant *E. coli* and the C7(−) and C7hm723(−) strains of *C. diphtheriae*. In addition, we demonstrate that the binding of DtxR to a diphtheria *tox* promoter/operator probe requires the addition of Mn^{2+} to the reaction mixture; however, binding may be blocked by addition of the chelator 2,2′-dipyridyl, anti-DtxR antiserum, and excess unlabeled probe to the reaction mixture. Deletion of one of the 9-base-pair inverted repeat sequences from the *tox* operator results in a loss of DtxR binding. The results presented here demonstrate that regulation of diphtheria toxin expression by DtxR requires direct interaction between this regulatory factor and the *tox* operator in the presence of a divalent heavy metal ion.**

While the structural gene for diphtheria toxin, *tox*, is carried by a family of closely related corynebacteriophages, the regulation of *tox* expression in *Corynebacterium diphtheriae* is mediated by a bacterial host determinant (1, 2). Boyd *et al.* (3) have recently reported molecular cloning and DNA sequence analysis of the *dtxR* gene from genomic libraries of the nonlysogenic nontoxigenic C7(−) strain of *C. diphtheriae*. DtxR has a deduced molecular weight of 25,316 and has been shown to regulate the expression of β-galactosidase from a *tox* promoter/operator (*toxPO*)–*lacZ* transcriptional fusion in recombinant *Escherichia coli*. The DtxR-mediated regulation of *tox* expression in *C. diphtheriae*, as well as in recombinant *E. coli*, has been shown to be dependent on the concentration of iron in the growth medium (3, 4). Schmitt and Holmes (5) and Boyd *et al.* (6) have recently demonstrated by molecular cloning of an iron-insensitive mutant *dtxR* allele from the C7hm723(−) strain of *C. diphtheriae* that DtxR from C7hm723 fails to regulate the expression of β-galactosidase from *toxPO–lacZ* transcriptional fusions.

The recent studies of Boyd and colleagues (3, 6) and Schmitt and Holmes (5, 7) have provided additional support for the hypothesis that the regulation of *tox* expression is regulated by a corynebacterial-determined iron-binding repressor as initially postulated by Murphy and Bacha (8). This model predicted DtxR to be an aporepressor, which in the presence of iron would form a complex that binds to the *tox* operator and blocks transcription. The putative *tox* operator is a 9-base-pair (bp) inverted repeat sequence that is separated by 9 bp (9–12). The putative operator overlaps both the −10 region of the *tox* promoter and the transcriptional start sites at −45, −40, and −39 upstream of the diphtheria toxin structural gene (13).

In the present contribution, we have recloned *dtxR* in the pET11c expression vector in order to direct the production of DtxR from the T7 promoter in *E. coli* HMS174(DE3)pDR1. In this host vector system, expression is directed by the T7 promoter on pET11 and T7 RNA polymerase, which is encoded by the lysogenic DE-3 strain of coliphage λ (14). After expression and purification of DtxR, we have determined the partial N-terminal sequence of the purified protein in order to ensure its identity with the amino acid sequence deduced from the *dtxR* structural gene. In addition, we have produced antibodies to DtxR and show by Western blot analysis that a single M_r 28,000 protein in crude extracts of both recombinant *E. coli* and the C7(−) and C7hm723(−) strains of *C. diphtheriae* is immunoreactive. Finally, we demonstrate by gel-shift analysis that purified DtxR specifically interacts with the *tox* operator and that association between DtxR and the *tox* operator is dependent on elevated concentrations of Mn^{2+} in the reaction mixture. We also show that the association between DtxR and the *tox* operator may be blocked and/or supershifted by the presence of anti-DtxR antiserum.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Medium. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in either Luria broth (LB) or LB agar medium supplemented with ampicillin (100 μg/ml). *C. diphtheriae* strains were grown in PTY medium [10 g of Casamino acids/2 g of yeast extract/10 ml of 1% L-tryptophan/2 ml of solution II (18)/1 ml of solution III (18), pH 7.2, per liter]. Before use, 30 ml of a sterile 50% maltose/0.5% $CaCl_2$ solution per liter of medium was aseptically added.

Nucleic Acids. Plasmid DNA was prepared by the alkaline lysis method and purified by CsCl/ethidium bromide centrifugation according to standard methods (19). Restriction endonucleases, DNA ligase, and Klenow fragment (Bethesda Research Laboratories) were used according to the specifications of the manufacturer. Restriction endonuclease frag-

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; PVDF, poly(vinylidene difluoride).

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Table 1. Bacterial strains, plasmids, and coliphage used in this study

Strain	Genotype	Ref. or source
E. coli		
JM101	*supE thi (lac-proAB)* [F′ *proAB*$^+$ *lacI*^{q}*Z* M15]	Ref. 15
DH5α	F$^-$(80d *lacZ* M15) (*lacZYA–argF*) U169 *recA1 endA1 hsdR17* (rk$^-$, mk$^-$) *supE44 thi1 gyrA relA1*	BRL
HMS174(DE3)		Ref. 14
Plasmid		
pHH2500	*amp*^{r} *dtxR*$^+$	Ref. 3
pRS551toxPO	*amp*^{r} *toxPO–lacZ*	Ref. 3
pDR1	*amp*^{r} *dtxR*$^+$	This work
Coliphage		
M13		Ref. 15
M13dtxR		This work
M13dtxRT7		This work
C. diphtheriae		
C7(−)	*tox*$^-$	Ref. 16
C7hm723(−)	*dtxR*	Ref. 17

ments were electrophoresed in 1% agarose gels in TBE (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.3). Oligonucleotides were synthesized on an Applied Biosystems PCR-Mate synthesizer and purified on Nensorb Prep cartridges (New England Nuclear) as directed by the manufacturer. To anneal complementary strands, equimolar concentrations of each strand were mixed in the presence of 100 mM NaCl, heated to 90°C for 10 min, and allowed to cool slowly to room temperature.

Oligonucleotide site-directed mutagenesis was performed by using a T7-Gen *in vitro* mutagenesis kit (United States Biochemical). DNA sequencing was performed by the dideoxynucleotide chain-termination method of Sanger *et al.* (20) as modified by Kraft *et al.* (21) using Sequenase (United States Biochemical).

Expression and Purification of DtxR. Expression of DtxR was directed from the T7 promoter on a derivative of pET11c as described by Studier *et al.* (14). *E. coli* HMS174(DE3)-pDR1 was grown at 37°C in LB medium with ampicillin at 100 μg/ml. When the absorbance (A_{600}) reached 0.8, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM to induce the expression of T7 RNA polymerase and subsequent transcription from the T7 promoter. Two hours after induction, the bacteria were harvested by centrifugation and frozen at −70°C overnight. The frozen cell pellet was resuspended in 10 mM Tris·HCl (pH 7.5), and the bacteria were lysed by sonication. The lysate was centrifuged at 25,000 × *g* for 20 min at 4°C to remove cellular debris. The clarified lysate was then applied to a DE-53 (Whatman) anion-exchange column equilibrated with 10 mM Tris·HCl (pH 7.5) and eluted with a linear gradient of 40–300 mM NaCl in the same buffer at 4°C. The fractions containing DtxR were monitored by SDS/PAGE and a gel electrophoresis mobility-shift assay.

Production of Antisera to DtxR. To produce anti-DtxR antibody, DtxR was further purified by using preparative SDS/PAGE. The relative position of the M_r 28,000 protein band was localized by soaking the gel in 3 M KCl, and the protein band was then excised from the gel. After extraction and dialysis against 20 mM phosphate buffer (pH 7.4), DtxR was emulsified in Ribi adjuvant (Ribi Immunochem). A total of 50 μg of purified protein was then injected into multiple subcutaneous sites in each of two New Zealand rabbits. Subsequent immunizations were administered 3 weeks after the initial injections. Animals were bled from the ear vein 12 days after the second immunization. Antibody titers were measured by an ELISA.

Gel Electrophoresis and Immunoblot Analysis. SDS/PAGE was performed according to the method of Laemmli (22) using 12% gels in the presence of 0.1 M dithiothreitol. Proteins were stained with Coomassie brilliant blue or transferred to a poly(vinylidene difluoride) (PVDF) membrane (Millipore) for immunoblot analysis. Immunoblots were probed with a 1:3000 dilution of anti-DtxR antiserum followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega). Immunoblots were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate using the ProtoBlot AP system according to the manufacturer's specification (Promega).

Gel Electrophoresis Mobility-Shift Assay. The gel electrophoresis mobility-shift assay used in this study is similar to that described by de Lorenzo *et al.* (23). Binding of DtxR protein to the diphtheria *toxPO* probe was carried out in 16 μl of reaction mixture containing 20 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 40 mM KCl, 2 mM dithiothreitol, 125 μM $MnCl_2$, 10% (vol/vol) glycerol, 1 μg of poly(dI-dC), 5 μg of bovine serum albumin, purified DtxR, and 3–5 fmol of ^{32}P-labeled probe. The probe containing the diphtheria *toxPO* region was excised from plasmid pRS551toxPO by digestion with *Eco*RI and *Bam*HI and labeled with [^{32}P]dATP by filling with Klenow fragment according to standard procedures. In the competition assays, unlabeled probe was added to the reaction mixture 10 min before addition of the labeled probe. In antibody neutralization assays, reaction mixtures were preincubated with antiserum for 2 hr at 4°C before addition of the *toxPO* probe. After the labeled probe was added, the mixture was incubated for 15 min at 25°C. Eight microliters of the reaction mixture was then applied to a 6% polyacrylamide gel in 40 mM BisTris borate, pH 7.5/125 μM $MnCl_2$/2.5% glycerol, and electrophoresed in the same buffer without glycerol at a constant voltage (200 V). After electrophoresis, the gels were dried and analyzed by autoradiography.

RESULTS

To direct the production of DtxR from the T7 promoter in recombinant *E. coli*, the *dtxR* structural gene was recloned into coliphage M13mp19, mutagenized to introduce a unique *Nde* I restriction site, and then recloned into the pET11c vector. As shown in Fig. 1, the *Pvu* II fragment encoding *dtxR* was purified from pHH2500 and blunt-end ligated into the *Sma* I site of the replicative form of M13mp19. A *Nde* I restriction endonuclease site was then introduced by the insertion of a T in the sequence CAATG to yield CATATG. In this sequence, the ATG is the translational initiation signal for the *dtxR* structural gene. After confirmation of the introduction of the *Nde* I site by nucleic acid sequencing, the *Nde* I/*Bam*HI fragment from M13dtxRT7 was then recloned into the *Nde* I and *Bam*HI sites of the pET11c expression vector to yield pDR1.

After ligation and transformation, several clones of *E. coli* HMS174(DE3) were isolated that contained plasmids with the restriction endonuclease digestion patterns expected for plasmid pDR1. One of these strains was selected and used for production of DtxR. The production of DtxR in HMS174(DE3)pDR1 is under the direction of the T7 promoter. After addition of IPTG, the structural gene for T7 polymerase, which is carried on the lysogenic DE3 derivative of coliphage λ, is induced and stimulates the expression of genes under the control of the T7 promoter. As shown in Fig. 2, SDS/PAGE analysis of crude extracts of HMS174(DE3)-pDR1 shows that *dtxR* is not expressed before addition of IPTG to the growth medium (lane 2); however, 2 hr after the addition of IPTG a major protein band with an electropho-

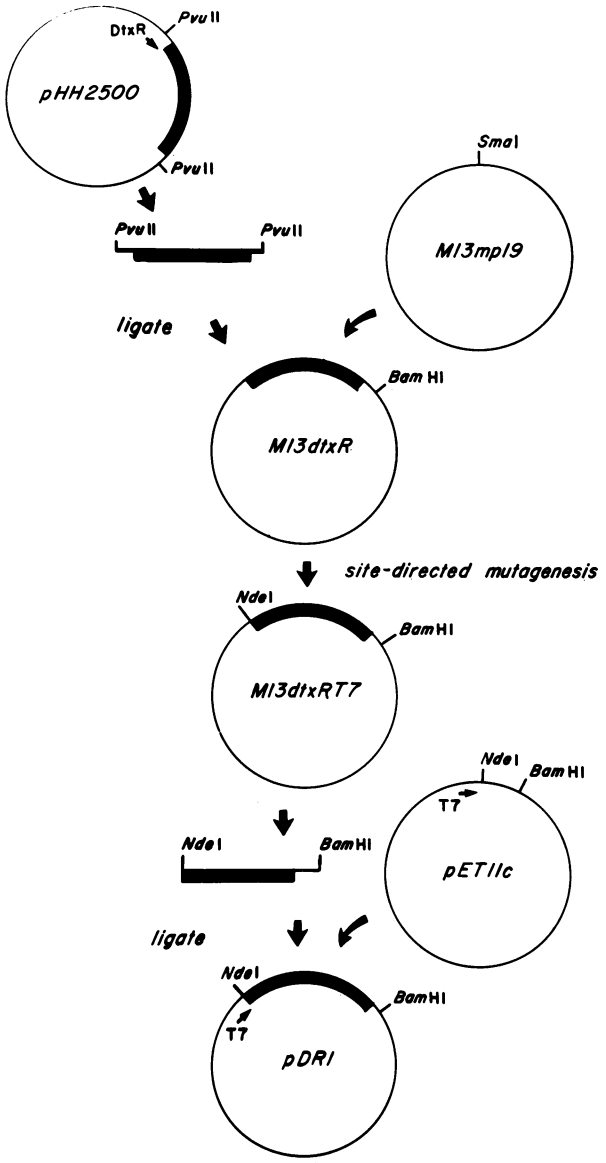

FIG. 1. Plasmid constructions used to place expression of *dtxR* under the control of the T7 promoter on pDR1.

retic mobility corresponding to M_r 28,000 may be seen (lane 3). Densitometric analysis of Coomassie blue-stained SDS/polyacrylamide gels suggests that after a 2-hr induction, DtxR accounts for 20–30% of the total cellular protein.

After induction and incubation of HMS174(DE3)pDR1 for 2 hr, the bacteria were harvested by centrifugation and lysed by sonication. The crude extract was centrifuged at 25,000 × *g* for 20 min to remove whole cells and bacterial debris and the soluble fraction was then chromatographed on DE-53 anion-exchange medium. The column was extensively washed and bound proteins were eluted with a linear NaCl gradient. As shown in Fig. 2, DtxR may be purified to >90% after anion-exchange chromatography.

To ensure that the M_r 28,000 protein was DtxR, the protein was electrophoretically transferred to a PVDF membrane for direct protein sequencing. A partial N-terminal amino acid sequence was then determined by sequential Edman degradation in an Applied Biosystems 470A protein sequencer. The partial sequence of the M_r 28,000 protein was found to be Met-Lys-Asp-Leu-Val-Asp-Thr-Thr-Glu-Met (J. O'Loughlin, personal communication). This sequence corresponds exactly with the N-terminal amino acid sequence of DtxR as

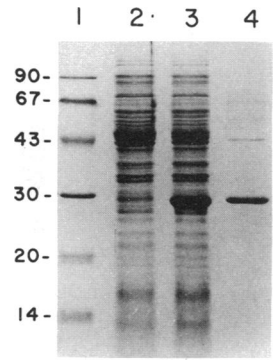

FIG. 2. SDS/PAGE analysis of total protein from *E. coli* HMS174(DE3)pDR1 before (lane 2) and 2 hr after (lane 3) induction with IPTG. Lane 4, DtxR peak fraction after anion-exchange chromatography on DE-53. Lane 1, molecular weight standards ($M_r \times 10^{-3}$). SDS/polyacrylamide gels were electrophoresed in the presence of 0.1 M dithiothreitol.

deduced from the nucleic acid sequence of the *dtxR* structural gene (3).

After DE-53 chromatography, DtxR was further purified by preparative PAGE. The M_r 28,000 protein band was excised from the gel and eluted in phosphate buffer, and then the protein was mixed with Ribi adjuvant and used to immunize New Zealand rabbits. After primary and secondary immunizations, serum was prepared and used to probe immunoblots of total protein extracts from a variety of strains of *E. coli* and *C. diphtheriae*. As shown in Fig. 3, a single M_r 28,000 protein band was found to be immunoreactive with anti-DtxR in both the wild-type C7(−) and mutant C7hm723(−) *dtxR* strains of *C. diphtheriae*, as well as *E. coli* HMS174(DE3)pDR1. In contrast, immunoblots of crude extracts of the HSM174(DE3) strain of *E. coli*, which does not carry the *dtxR* structural gene, are negative. Immunoblots probed with preimmune serum were also found to be negative (data not shown).

Earlier studies have suggested that regulation of diphtheria toxin expression was mediated by a corynebacterial-negative controlling element (3–5, 8, 9, 12). Since the expression of diphtheria toxin in toxigenic *C. diphtheriae* and β-galactosidase from a *tox* promoter/operator–*lacZ* transcriptional fusion in recombinant *E. coli* pHH2500 is sensitive to repression by excess iron in the growth medium (3, 4), it was postulated that binding of DtxR to the putative diphtheria *tox* operator required iron, or other divalent heavy metal ions, as shown by Groman and Judge (24). To examine the interaction between DtxR and the putative *tox* operator, we have used a

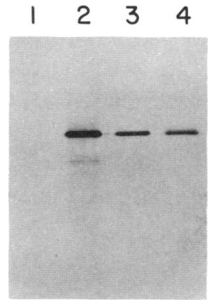

FIG. 3. Immunoblot analysis of crude protein extracts of *E. coli* and *C. diphtheriae* strains. After SDS/PAGE separation in the presence of 0.1 M dithiothreitol, total protein was transferred to PVDF membranes and probed with rabbit anti-DtxR antiserum. Lanes: 1, *E. coli* HMS174(DE3) (150 ng); 2, *E. coli* HMS174(DE3)-pDR1 (10 ng); 3, *C. diphtheriae* C7(−) (150 ng); 4, *C. diphtheriae* C7hm723(−) (150 ng).

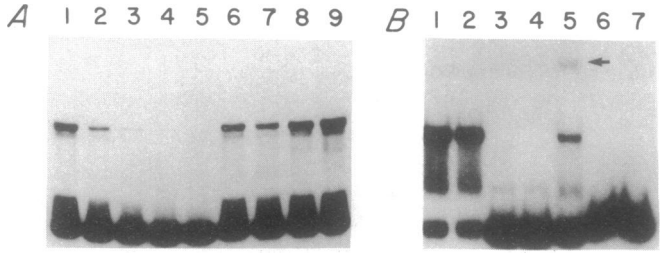

FIG. 4. Gel mobility-shift assay of DtxR binding to ^{32}P-labeled *toxPO* probe in the presence and absence of inhibitors. (*A*) Lanes: 1, DtxR control; 2, 8 fmol of unlabeled *toxPO* probe; 3, 40 fmol of unlabeled *toxPO* probe; 4, 200 fmol of unlabeled *toxPO* probe; 5, 1000 fmol of unlabeled *toxPO* probe; 6, 8 fmol of *toxPO-C1* probe; 7, 40 fmol of unlabeled *toxPO-C1* probe; 8, 200 fmol of unlabeled *toxPO-C1* probe; 9, 1000 fmol of unlabeled *toxPO-C1* probe. (*B*) Lanes: 1, DtxR control; 2, 1:160 dilution of preimmune serum; 3, 1:160 dilution of anti-DtxR antiserum; 4, 1:480 dilution of anti-DtxR antiserum; 5, 1:1440 dilution of anti-DtxR antiserum; 6, probe alone; 7, DtxR in the presence of 300 μM 2,2'-dipyridyl. Arrow indicates a supershift band in the presence of low concentrations of anti-DtxR antiserum.

gel electrophoresis mobility-shift assay. As shown in Fig. 4, the interaction between DtxR and the *toxPO* probe is specific. In the absence of inhibitors, the binding of DtxR to the probe requires addition of the divalent cation Mn^{2+} to the reaction mixture (Fig. 4*A*, lane 1). In the absence of a divalent heavy metal ion, DtxR fails to bind to the *toxPO* probe (data not shown). Moreover, addition of the chelator 2,2'-dipyridyl to the reaction mixture was found to abolish DtxR binding to the *toxPO* probe (Fig. 4*B*, lane 7). The binding of DtxR to the probe is not blocked by addition of preimmune serum to the reaction mixture (lane 2); however, binding is blocked by the addition of anti-DtxR antiserum (lane 3). The specificity of the interaction between DtxR and the *tox* operator probe is also shown by the supershift of the DtxR–*tox* operator probe complex after incubation with a 1:1440 dilution of the anti-DtxR antiserum (lane 5). The supershifted complex is most likely to have the following composition: anti-DtxR/DtxR/Mn^{2+}/^{32}P-labeled *toxPO*.

Since many operators exhibit dyad symmetry, we examined the role played by the inverted repeat sequences in the putative *tox* operator in the binding of DtxR. A deletion mutant form of the diphtheria *toxPO*, *toxPO-C1*, was constructed in which the downstream inverted repeat sequence was replaced with unrelated sequences as shown in Fig. 5. As shown in Fig. 4*A* (lanes 6–9), addition of unlabeled *toxPO-C1* probe fails to block the interaction between DtxR and the ^{32}P-labeled *toxPO* probe. In contrast, addition of unlabeled *toxPO* probe to the reaction mixture was found to competitively inhibit the interaction between DtxR and the labeled probe (lanes 2–5).

DISCUSSION

To facilitate purification of DtxR from recombinant *E. coli*, we have introduced a unique *Nde* I restriction site immediately upstream of the *dtxR* ATG initiation codon and then recloned the structural gene into the pET11c vector. In the HMS174(DE3) strain of *E. coli*, T7 RNA polymerase is encoded on a lysogenic strain of coliphage λ under the control of *lacI* (14). We show that after addition of IPTG and a 2-hr incubation, the final yield of DtxR in the cytoplasmic fraction is 20–30% of total protein. DtxR may be purified from crude lysates of HMS174(DE3)pDR1 to ≥90% by anion-exchange chromatography on Whatman DE-53.

DtxR was further purified by preparative SDS/PAGE. New Zealand rabbits were then immunized with protein, which was eluted from the gel and emulsified in Ribi adjuvant. The resulting hyperimmune serum was used to probe immunoblots of recombinant *E. coli* and the C7(−) and C7hm723(−) strains of *C. diphtheriae*. While *E. coli* HMS174(DE3) was found to be immunoblot negative, a single M_r 28,000 protein band was found to be immunoblot positive in *E. coli* HMS174(DE3)pDR1, as well as the *C. diphtheriae* strains C7(−) and C7hm723(−).

The regulation of diphtheria toxin expression in *C. diphtheriae* strains has been shown to be directed at the level of transcription (25). Moreover, the isolation of both corynebacterial (17) and corynebacteriophage (26, 27) mutants in which *tox* expression is insensitive to the level of exogenous iron strongly suggested that the regulation of *tox* was mediated through an iron-binding negative control element as proposed by Murphy and Bacha (8). The recent studies of Boyd *et al.* (3), who have cloned, sequenced, and studied the *in vivo* properties of *dtxR* by using a diphtheria *toxPO–lacZ* transcriptional fusion recombinant *E. coli*, as well as those of Schmitt and Holmes (7), who have cloned and studied the *in vivo* properties of *dtxR* in mutant strains of *C. diphtheriae*, have added additional support to the model of *tox* regulation as proposed by Murphy and Bacha (8).

We have further extended these observations by demonstrating that DtxR selectively binds to a *toxPO* probe in a gel electrophoresis mobility-shift assay. In this assay system, we show that binding of DtxR to the probe requires the addition of Mn^{2+} to the reaction mixture; we have used Mn^{2+}, rather than Fe^{2+}, as the divalent heavy metal ion in activation of DtxR because of its stability in aerobic conditions. Moreover, it should be noted that Groman and Judge (24) have previously shown that the addition of Mn^{2+} to cultures of lysogenic and toxigenic *C. diphtheriae* also repressed the expression of diphtheria toxin. The specificity of the interaction between DtxR and the labeled *toxPO* probe in the presence of Mn^{2+} is demonstrated by competitive inhibition of the interaction after addition of unlabeled probe. Furthermore, the addition of anti-DtxR antiserum to the reaction mixture also blocked DtxR binding to the *toxPO* probe. At a 1:1440 dilution of anti-DtxR antiserum, we show that DtxR/Mn^{2+}/^{32}P-labeled *toxPO* is supershifted, presumably by anti-DtxR antibody binding to the complex.

Since many operators require dyad symmetry, we have examined the effect of mutant probe *toxPO-C1* when added as a competitive inhibitor in the mobility-shift assay system. As shown in Fig. 4*A*, the *toxPO-C1* probe fails to compete with ^{32}P-labeled *toxPO* for DtxR binding. The results of this experiment were anticipated since β-galactosidase expression from the *toxPO-C1–lacZ* transcriptional fusion was not

toxPO

5'-ACC CTT ATA ATT AGG ATA GCT TTA CCT AAT TAT TTT-3'

toxPO-C1

5'-ACC CTT ATA ATT AGG ATA GCT TTA AGC CCG GGT TTT-3'

FIG. 5. Partial sequence of the *tox* promoter operator probes used in this study.

regulated by DtxR in recombinant *E. coli* (J.B. and J.R.M., unpublished data). These experiments provide additional support to the hypothesis that the 9-bp inverted repeat sequences in the *tox* regulatory region are, in fact, the *tox* operator.

Fourel *et al.* (12) have shown that a protein designated DtoxR, found in crude extracts of *C. diphtheriae*, could specifically interact with the presumptive *tox* operator and protect it from DNase digestion. Since these experiments were conducted with crude extracts rather than a purified protein, it is difficult to postulate whether DtoxR and DtxR are identical. Nonetheless, when the gel mobility-shift assay results described above are taken into consideration, the demonstration that anti-DtxR antiserum is immunoreactive with a single M_r 28,000 protein in crude extracts of both recombinant *E. coli* and *C. diphtheriae* strains C7(−) and C7hm723(−) suggests that DtoxR and DtxR may be identical. To rigorously address this question, the DNA footprints of DtxR purified from HMS174(DE3)pDR1 can be compared with the serologically related protein purified from *C. diphtheriae*.

Schmitt and Holmes (7) have recently shown that DtxR is able to function as a global regulatory element and, in addition to the *tox* operon, appears to control the expression of several siderophore genes in *C. diphtheriae* in a manner analogous to Fur in *E. coli*. While the Fur and DtxR operator sequences have also been shown to be similar (28), Boyd *et al.* (3) have shown that DtxR is unable to control expression of the iron-regulated outer membrane proteins in *E. coli*. While the fine details that involve the stoichiometry and sites of interaction between DtxR, a divalent heavy metal ion, and the *tox* operator remain to be determined, the underlying model of diphtheria *tox* regulation as proposed by Murphy and Bacha (8) has now been shown to be essentially correct.

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