

# Regulation of cyclic peptide biosynthesis in a plant pathogenic fungus by a novel transcription factor

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Communicated by Hans J. Kende, Michigan State University, East Lansing, MI, September 18, 2001 (received for review August 5, 2001)

**Strains of the filamentous fungus *Cochliobolus carbonum* that produce the host-selective compound HC-toxin, a cyclic tetrapeptide, are highly virulent on certain genotypes of maize (*Zea mays* L.). Production of HC-toxin is under the control of a complex locus, *TOX2*, which is composed of at least seven linked and duplicated genes that are present only in toxin-producing strains of *C. carbonum*. One of these genes, *TOXE*, was earlier shown to be required for the expression of the other *TOX2* genes. *TOXE* has four ankyrin repeats and a basic region similar to those found in basic leucine zipper (bZIP) proteins, but lacks any apparent leucine zipper. Here we show that *TOXE* is a DNA-binding protein that recognizes a ten-base motif (the "tox-box") without dyad symmetry that is present in the promoters of all of the known *TOX2* genes. Both the basic region and the ankyrin repeats are involved in DNA binding. A region of *TOXE* that includes the first ankyrin repeat is necessary and sufficient for transcriptional activation in yeast. The data indicate that *TOXE* is the prototype of a new family of transcription factor, so far found only in plant-pathogenic fungi. *TOXE* plays a specific regulatory role in HC-toxin production and, therefore, pathogenicity by *C. carbonum*.**

Interactions between plants and their pathogens frequently show a high degree of specificity, in which single genes in the host and the pathogen control whether the interaction will result in susceptibility or resistance (1). Among the known agents of specificity are the host-selective toxins, low-molecular-weight compounds produced by certain fungal pathogens, notably species of *Cochliobolus* and *Alternaria* (2, 3). In the interaction between the filamentous fungus *Cochliobolus carbonum* and its host, maize, specificity is controlled by HC-toxin, a cyclic peptide host-selective toxin with the structure cyclo-(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxy-8-oxodecanoic acid. Disease occurs only when HC-toxin-producing (*Tox2*<sup>+</sup>) isolates of *C. carbonum* infect maize plants that are homozygous recessive at the *Hm1* and *Hm2* loci. These genes encode NADPH-dependent reductases that confer insensitivity to HC-toxin, and hence resistance to *C. carbonum*, by reducing the 8-carbonyl group of Aeo (4–6).

HC-toxin, like other Aeo-containing cyclic tetrapeptides such as trapoxin (7), is a potent inhibitor of histone deacetylases from maize and other organisms (8, 9). By a mechanism that remains to be elucidated, inhibition of histone deacetylase during the early stages of infection permits *C. carbonum* to infect and colonize maize plants (10).

HC-toxin production by *C. carbonum* is controlled by a complex genetic locus called *TOX2* (3). *TOX2* contains at least seven genes with an established or putative role in HC-toxin biosynthesis. *HTS1* encodes a 570-kDa nonribosomal peptide synthetase (11, 12). *HTS1* is tightly clustered with *TOX4*, which encodes a putative HC-toxin efflux carrier (13). *TOXC* encodes a fatty acid synthase beta subunit that is proposed to be involved in the biosynthesis of the side chain of Aeo (14). *TOXD* is predicted to encode a dehydrogenase, and although its role in HC-toxin biosynthesis has not been established, its amino acid sequence is most closely related to that of *lovC*, which is required for biosynthesis of the polyketide lovastatin by *Aspergillus terreus* (15). *TOXF* encodes a putative branched-chain amino acid

transaminase and is closely clustered with *TOXG*, which encodes an alanine racemase (16, 17). All of the known *TOX2* genes are present in multiple, linked copies in all *Tox2*<sup>+</sup> isolates that have been studied, and are completely absent from toxin nonproducing (*Tox2*<sup>-</sup>) isolates (17, 18).

The seventh known gene of the *TOX2* cluster is *TOXE*. *TOXE* is required for HC-toxin biosynthesis and for mRNA expression of the other genes of *TOX2*. *TOXE* mutants display no phenotype other than loss of HC-toxin production and virulence, and thus *TOXE* appears to be a specific regulator of HC-toxin biosynthesis (19). The product of *TOXE* (*TOXE*) contains two motifs that are individually widespread in nature but which had not previously been found together in the same protein (Fig. 1). An amino acid sequence at its N terminus matches the consensus sequence of the basic DNA binding domain of the leucine zipper (bZIP) family of DNA-binding transcriptional regulators (20). However, *TOXE* does not contain a leucine zipper, a feature essential to the function of true bZIP proteins. At its C terminus *TOXE* is predicted to contain four repeats of the ankyrin motif (Fig. 1). This motif is found in a number of different types of proteins and has been shown to mediate protein–protein interactions (21, 22).

From its requirement for expression of other *TOX2* genes and its possession of a predicted DNA binding motif, it seemed possible that *TOXE* might be a transcription factor that coordinates expression of the *TOX2* genes. However, its overall unusual structure compared with other known proteins, including known transcription factors, left its precise function unclear. In this study, we demonstrate that *TOXE* is a transcription factor that binds as a monomer or homodimer to a specific nonpalindromic DNA sequence that is present in one or two copies within the promoters of the known *TOX2* genes. In addition to its ability to bind DNA, *TOXE* has intrinsic ability to activate transcription in yeast.

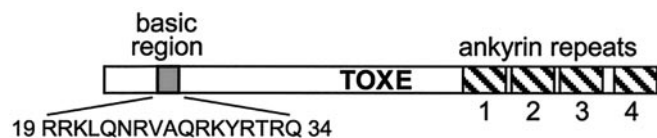
## Materials and Methods

**Expression of *TOXE* in *Escherichia coli*.** The plasmid pAJ39 was used as a source of a *TOXE* cDNA (GenBank accession no. AF038874; ref. 19). The 5' end of *TOXE* was amplified by PCR using the primers JDW623 (5'-CACGGATCCGGCAGCACTTCCCCGAATAGC-3') and JDW624 (5'-CCTTACGCTGCTAGTTACGAAGC-3') to introduce a *Bam*HI site (italicized). The 237-bp product was digested with *Bam*HI and *Sty*I and used to replace the 5' end of the *TOXE* cDNA insert in pAJ39, creating plasmid pKP9. The *TOXE* cDNA insert was removed from pKP9 with *Bam*HI and *Kpn*I and cloned into the *Bam*HI and *Kpn*I sites of the bacterial expression vector pQE30 (Qiagen, Chatsworth, CA) to create the *TOXE* expression vector pKP10. Plasmids pKP36 and pKP37 were constructed for expressing C-terminal deletions of *TOXE* in *E. coli* by using either

Abbreviations: GAL4<sub>DBD</sub>, GAL4 DNA binding domain; UTR, untranslated region; bZIP, basic leucine zipper.

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**Fig. 1.** Structure of TOXE. TOXE has a molecular mass of 49 kDa and 441 aa. The bZIP-like basic domain is between residues 19 and 34 and the four ankyrin repeats are located between residues 290 and 315, 323 and 350, 357 and 384, and 415 and 441 (19).

a *Bam*HI–*Eco*RV or *Bam*HI–*Eco*RI fragment from pKP10 cloned into pQE30, respectively. For expression, the *E. coli* strain M15 containing pREP4 (Qiagen) was transformed with pKP10. A sample of an overnight culture (250  $\mu$ l) was used to inoculate 5 ml of 2  $\times$  YT medium (16 g/l tryptone/10 g/l yeast extract/5 g/l NaCl, pH 7.5) containing ampicillin and kanamycin. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; 0.1 mM) was added after 2.5 h of growth, and the cells were grown for an additional 30 min before harvest.

**Determination of Transcriptional Start Sites.** Random amplification of cDNA ends (RACE), using a kit from Life Technologies (Rockville, MD), was used to determine the length of the 5' untranslated regions (5'-UTRs) of *TOXC* and *TOXD* (17, 23).

**Southwestern Blot Analysis.** *E. coli* cell pellets were resuspended in sample buffer [0.25 M Tris-HCl, pH 6.8/20% 2-mercaptoethanol (vol/vol)/8% SDS (wt/vol)/30% sucrose (wt/vol)/0.01% bromophenol blue] and boiled for 5 min. The denatured protein extracts were fractionated by SDS/PAGE (10% acrylamide). After equilibration with blotting buffer [25 mM Tris/192 mM glycine/20% methanol (vol/vol)] the proteins were transferred to nitrocellulose (Schleicher & Schuell) by using a Mini *Trans*-Blot cell (Bio-Rad). The membrane was incubated in renaturation buffer (100 mM Hepes, pH 7.5/100 mM KCl/1 mM DTT/0.1 mM EDTA/10 mM MgCl<sub>2</sub>/5% nonfat milk) at 4°C for 18 h. After rinsing with TNE-50 buffer (10 mM Tris, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM DTT), the blot was incubated in the same buffer containing a [<sup>32</sup>P]-labeled DNA probe (10<sup>6</sup> cpm/ml) and nonspecific competitor DNA (10 mg/ml sheared salmon sperm DNA) for 6 h at 25°C. The blot was then washed two times with TNE-50 at 25°C for 15 min followed by autoradiography.

**Construction and Labeling of Probes for Southwestern Blotting.** Gene promoter fragments were amplified by PCR, using specific primers for each of the promoters. Primers were 18–26 nt in length (sequences available on request). PCR conditions were 35 cycles (94°C for 1 min; 55°C for 2 min; 72°C for 1 min) after an initial denaturation for 2 min at 94°C, followed by a final extension of 5 min at 72°C. Reaction products were purified by gel filtration chromatography and labeled with  $\alpha$ -[<sup>32</sup>P]dCTP by random priming (24). To make double-stranded probes, complementary oligonucleotides JDW-831 (5'-AACACAATCTCACGTAAGTCTGCAG-3') and JDW-832 (5'-CCTGCAGACTTACGTGAGATTGTGT-3') representing a TOXE recognition element (tox-box) from the *TOXA* promoter, and JDW-833 (5'-AACACAGCAGGACGTAAGTCTGCAG-3') and JDW-834 (5'-CCTGCAGACTTACGTCTGCTGTGT-3'), representing a mutant version of this site, were annealed before use. Double-stranded oligonucleotides were end-labeled with  $\gamma$ -[<sup>32</sup>P]ATP by using polynucleotide kinase (24).

**Yeast Strains and Methods.** Standard methods were used (25). Yeast strain MG106 (MATa *ade2-1 can1-100 his 3-11 15 leu 2-3 112 trp 1-1 ura 3-1*; ref. 26) was used for testing DNA binding, and Y190 (MATa, *ura3-52, his3-200, hys2-801, ade2-101, trp1-901, leu2-3,*

*112, gal4 $\Delta$ , gal80 $\Delta$ , cyh<sup>r</sup>2, LYS2::GAL1<sub>UAS</sub>-HIS3<sub>TATA</sub>-HIS3, URA::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ*; CLONTECH) for testing TOXE transcriptional activation function.

**Analysis of DNA Binding in Yeast.** Complementary oligonucleotides containing either four tandem copies of the wild-type TOXE recognition sequence (tox-box) (JDW-825, 5'-tcgaATCTCACGTAATCTCACGTAATCTCACGTAATCTCACGTA-3', and JDW-823, tcgaTACGTGAGATTACGTGAGATTACGTGAGATTACGTG-3'; lowercase letters indicate *Xho*I overhangs used for cloning) or mutant versions (JDW-836, 5'-tcgaGCAGGACGTAGCAGGACGTAGCAGGACGTAGCAGGACGTA-3', and JDW-837, 5'-tcgaTACGTCTGC-TACGTCTGCTACGTCTGCTACGTCTGC-3') were annealed and ligated into the *Xho*I site of the *lacZ* reporter vector pBgl-lacZ (27). The resulting reporter constructs were integrated into the *ura3* locus of strain MG106 by transformation and selection for uracil prototrophy.

TOXE was expressed in yeast by using the pG-1 expression vector (28) containing the TOXE cDNA from pAJ39 modified by PCR to create restriction sites for cloning. The resulting plasmid pKP40 was transformed into the reporter strains by selecting for tryptophan prototrophy.

**Analysis of TOXE Activation Activity.** To test the activation activity of fragments of TOXE fused with the GAL4 DNA binding domain (GAL4<sub>DBD</sub>), *Nco*I sites (underlined) were introduced into TOXE at sites corresponding to amino acids 1, 167, and 254 by using PCR primers JDW-768 (5'-GCTGGATCCACACCATGGGCACGACTTCCCCG-3'), JDW-868 (5'-CGTGGATCCTGGCCATGGACTTGCCTTCTGTT-3'), and JDW-1059 (5'-GGAGGGATCCATGGATTCTAGTTATAGTAACCTC-3'), respectively. The carboxy termini of the fragments tested for activation activity were created by using native restriction sites or reverse primer JDW-1061 (5'-GGGGGTGCGACTAGTCTTCCTTTGGGCCATG-3') corresponding to amino acid 289. The resulting products were cloned into pAS2-1 (CLONTECH) to express GAL<sub>DBD</sub>:TOXE fusion proteins.

**Site-Directed Mutagenesis.** Amino acid substitutions were introduced into the TOXE basic region by using PCR primers containing the desired mutations (sequences available on request). The products were cloned into pKP9 by using restriction sites present in the TOXE sequence. All changes were confirmed by DNA sequencing. Amino acid changes in the activation region of TOXE were made by using the “megaprimer” mutagenesis protocol (29). All changes were confirmed by DNA sequencing. Mutated copies of TOXE were cloned into pAS2-1 and used to express GAL<sub>DBD</sub>:TOXE fusions in yeast strain Y190.

**$\beta$ -Galactosidase Assay.** Yeast transformants expressing  $\beta$ -galactosidase were assayed in two ways. For a qualitative assay, yeast cells were grown on SD plates for 2–3 days at 30°C and overlaid with a nitrocellulose membrane filter (Schleicher & Schuell) for 5 min. The nitrocellulose filter was frozen in liquid nitrogen and placed on a piece of filter paper saturated with 50 nM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) dissolved in Z-buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>/40 mM NaH<sub>2</sub>PO<sub>4</sub>/10 mM KCl/1 mM MgSO<sub>4</sub>/38 mM  $\beta$ -mercaptoethanol, pH 7.0). The filters were monitored for the appearance of blue color after incubation at 30°C for 1 h. For a quantitative assay, yeast cells from a 2-ml overnight culture in SD medium were used to inoculate 8 ml of fresh YPD medium. The cultures were grown at 28°C with shaking (230–250 rpm) until the cells reached a mid-log phase (OD<sub>600</sub> 0.3–0.5). Cells (1.5 ml) were harvested by centrifugation at 14,000 rpm for 30 sec and washed in Z-buffer. Cells were resuspended in 300  $\mu$ l of Z-buffer and disrupted with three freeze–thaw cycles. Aliquots of the disrupted cells (100  $\mu$ l) were

diluted with 700  $\mu$ l of Z-buffer and mixed with 160  $\mu$ l of 4 mg/ml *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) dissolved in Z-buffer. After incubation at 30°C for 10 min, 400  $\mu$ l of 1 M Na<sub>2</sub>CO<sub>3</sub> was added and the A<sub>410</sub> measured. One unit of  $\beta$ -galactosidase is defined as the amount which hydrolyzes 1  $\mu$ mol of ONPG per min per cell (30).

## Results

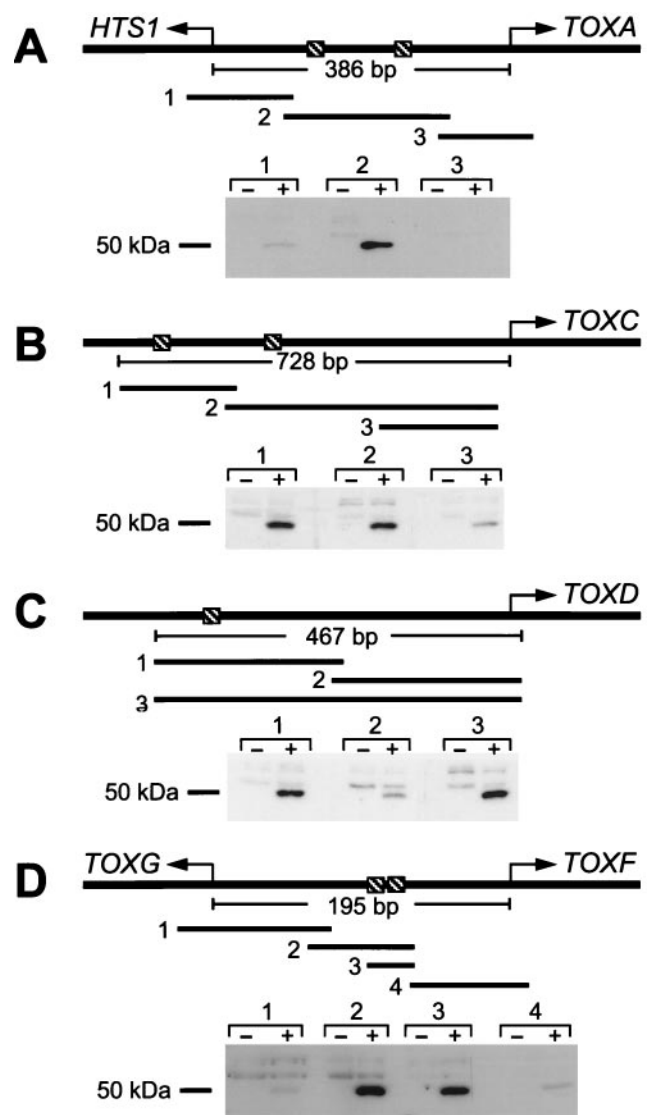
Before analysis of the interaction between TOXE and the *TOX2* promoters, it was essential to know the transcriptional start sites of the *TOX2* genes. *HTS1* and *TOXA* are clustered and divergently transcribed. Their 5'-UTRs are 162 and 145 bp, respectively (13). The 5'-UTR of *TOXD* was determined by random amplification of cDNA ends (RACE) to be 17 bp (Y. Cheng, K.F.P., and J.D.W., unpublished results). *TOXF* and *TOXG* are also clustered and divergently transcribed, and have 5'-UTRs of 59 and 46 bp, respectively (17). The 5'-UTR of *TOXC* was determined by RACE to be 167 bp (data not shown).

### TOXE Binds to a Specific Sequence in the Promoters of the *TOX2* Genes.

It was not possible to express TOXE in soluble form in *E. coli*, thereby precluding the analysis of DNA binding by gel mobility shift assays. TOXE was instead expressed in *E. coli* and analyzed by southwestern blotting (i.e., separation of proteins by SDS/PAGE followed by transfer to nitrocellulose, renaturation, and probing with double-stranded radiolabeled DNA fragments). For each *TOX2* gene promoter tested, one or more DNA fragments were found to bind strongly to a protein of the correct size in TOXE-expressing *E. coli* extracts (Fig. 2). Analysis of the sequences of the DNA fragments that bound to TOXE revealed the presence of a conserved nucleotide sequence, which was therefore a candidate for the TOXE recognition site. This 10-bp motif, which we refer to as the *tox-box*, is highly conserved at eight positions. Nucleotides at five of the positions are absolutely conserved, including the first four, ATCT (Fig. 3). Examination of the DNA sequences upstream and downstream of this element gave no indication that the *tox-box* sequence has dyad symmetry. The *tox-box* was present in all DNA fragments that bound TOXE and was absent from all fragments that did not bind TOXE (Fig. 2). The promoters of *HTS1/TOXA*, *TOXC*, and *TOXF/TOXG* contain two copies of the *tox-box* and *TOXD* contains one copy (Fig. 2). No evidence was found for a *tox-box* in the promoter of *TOXE*, suggesting that *TOXE* does not regulate itself. As further evidence for the importance of the *tox-box* for TOXE binding, changing the first five oligonucleotides of the *tox-box* eliminated binding of TOXE (Fig. 4).

**TOXE Functions as a Transcription Factor in Yeast.** To test the ability of TOXE to bind specifically to the *tox-box* *in vivo* as well as *in vitro*, a yeast expression system was used. Four copies of wild-type or mutant versions of the *tox-box* sequence, in both forward and reverse orientations, were cloned upstream of a *GAL1* promoter driving *lacZ* expression. This promoter is inactive by itself because of a lack of upstream activation sequences (ref. 27; Fig. 5A). Yeast strains containing the various *tox-box* constructs were then transformed with an expression plasmid containing *TOXE* driven by the constitutive yeast *GPD* promoter. Only strains containing both the wild-type *tox-box* and expressing *TOXE* had detectable levels of  $\beta$ -galactosidase activity (Fig. 5B). Both orientations of the wild-type *tox-box* were active (Fig. 5B).

**Identification of Regions of TOXE Important for DNA Binding.** In the bZIP family of proteins the basic region determines DNA binding-site specificity through direct contacts with DNA (20). To test whether this was also true for the basic region of TOXE, we analyzed this region by alanine scanning (31). Seven basic amino acids between residues 19 and 34 were changed to alanines and expressed in yeast strain KP50.1 (see Fig. 5). Only



**Fig. 2.** TOXE binds to the promoters of the *TOX2* genes. Total protein extracts from *E. coli* expressing (+) or not expressing (-) TOXE were separated by SDS/PAGE and transferred to nitrocellulose membranes. After renaturing the bound proteins, the membranes were probed with the indicated <sup>32</sup>P-labeled DNA fragments. Hatched boxes indicate *tox-box* sequences (see Fig. 3) relative to the transcriptional start sites and transcriptional directions of the *TOX2* genes, indicated by the arrows. Note that A–D are not drawn to scale.

strains expressing wild-type TOXE had detectable levels of  $\beta$ -galactosidase activity (Fig. 6A). Because failure to act *in vivo* could have been due to poor expression, reduced protein stability, or failure to localize to the nucleus, and not necessarily due to reduced DNA binding, all of the mutant constructs were also tested for their ability to bind to the *tox-box* by southwestern blotting. All of the mutant proteins were expressed in *E. coli* at levels comparable to the wild-type (Fig. 6B), but all had reduced ability to bind to the *tox-box* (Fig. 6B). Mutation of four of the highly conserved basic residues completely eliminated DNA binding (Fig. 6B).

The results of the mutational analysis of the TOXE basic region indicated that the basic region near the N terminus was essential for DNA binding but not necessarily sufficient. To test this, two carboxy-terminal deletion mutants, which eliminated three or four of the four ankyrin repeats, respectively, were tested for *in vitro* DNA binding. Both of the truncated versions



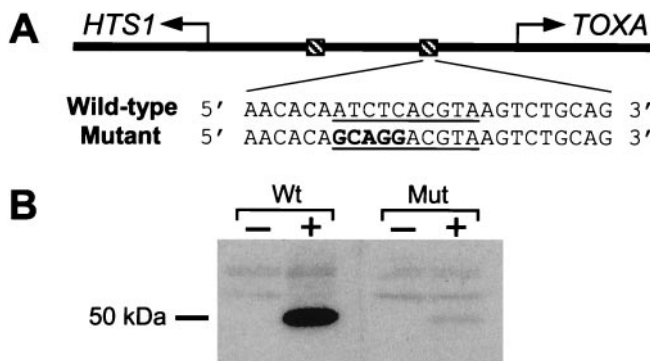
Promoter	TOXE binding site and location	
<i>TOXA/HTS1</i>	-169	ATCTCACGTA -160
<i>TOXA/HTS1</i>	-303	ATCTCACGCA -294
<i>TOXF/TOXG</i>	-92	ATCTCGCGTA -83
<i>TOXF/TOXG</i>	-112	ATCTTACGTA -103
<i>TOXC</i>	-222	ATCTCTCGTC -231
<i>TOXC</i>	-664	ATCTCTCGAA -655
<i>TOXD</i>	-195	ATCTCTAGGC -186
<b>"tox-box" consensus</b>	<b>ATCTCNGNA</b>	

**Fig. 3.** Sequences of the TOXE-binding sites from the promoters of the *TOX2* genes, as deduced from the results shown in Fig. 2 and from comparative sequence analysis. Sequence locations are relative to the transcriptional start sites. For the *HTS1/TOXA* and the *TOXF/TOXG* promoters, the locations are relative to *TOXA* and *TOXF*, respectively (see Fig. 2). Note that the first tox-box of *TOXC* is in the opposite orientation to the others.

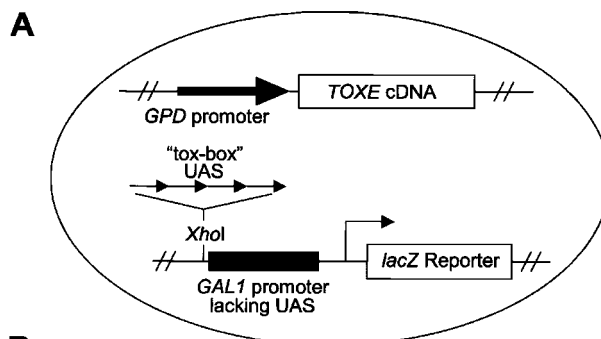
of TOXE were expressed in *E. coli* at levels comparable to wild-type TOXE (Fig. 7A), but neither was able to bind DNA (Fig. 7B).

**Identification of the Transcriptional Activation Region of TOXE.** When fused to the GAL4<sub>DBD</sub> and expressed in yeast strain Y190, TOXE by itself (i.e., without coexpressing the activation domain of GAL4) induced expression of *lacZ*, indicating that native TOXE has the capacity not only to bind DNA but also to activate transcription. To characterize the region or regions of TOXE responsible for activation, a series of amino- and carboxy-terminal deletions of TOXE were expressed as fusion proteins to GAL4<sub>DBD</sub> and tested for their ability to induce expression of *lacZ*. Of the TOXE fragments tested, the smallest one that could act as an activation domain encompassed amino acids 254–317 (Fig. 8). All active fragments contained this region, which extends from 35 aa in front of the first ankyrin repeat to one amino acid after. These results indicate that TOXE has a discrete activation domain and that, whereas activation possibly involves at least one of the ankyrin repeats, it does not require all four repeats.

The activation domain composed of amino acids 254–317 was further analyzed by alanine scanning. This region is not partic-



**Fig. 4.** Mutation of the conserved residues in the tox-box eliminate TOXE binding. (A) Representation of the *TOXA/HTS1* promoter. Hatched boxes indicate the two tox-boxes. (B) Double-stranded oligonucleotides containing the wild-type (Wt) tox-box sequence (underlined) and surrounding nucleotides, and a mutant version (Mut) in which all five of the highly conserved nucleotides were changed (indicated in bold lettering), were used as <sup>32</sup>P-labeled probes against southwestern blots. “–” Indicates total protein extracts from *E. coli* not expressing TOXE (control), and “+” indicates *E. coli* expressing TOXE.



**B**

Strain #	Upstream Activation Sequence		TOXE expression	β-galactosidase units
	tox-box	Orientation		
YKP50.1-E	Wild-type	Forward	+	38
YKP50.3-E	Wild-type	Reverse	+	69
YKP49.13-E	mutant	Forward	+	0
YKP49.14-E	mutant	Reverse	+	0
YKP50.1-pG-1	Wild-type	Forward	–	0
YKP50.3-pG-1	Wild-type	Reverse	–	0
YKP49.13-pG-1	mutant	Forward	–	0
YKP49.14-pG-1	mutant	Reverse	–	1

**Fig. 5.** TOXE acts as a sequence-specific DNA binding protein and transcriptional activator in yeast. (A) Four tandem copies of a double-stranded oligonucleotide containing either the wild-type or the mutant tox-box (see Fig. 4) were fused in both orientations upstream of GAL1 without upstream activating sequences (UAS) fused to *lacZ* (27). TOXE driven by the constitutive yeast GPD promoter was expressed from a plasmid. (B) Resulting β-galactosidase activities of the various yeast strains. “TOXE expression” indicates whether the yeast cells contained the plasmid expressing TOXE.

ularly rich in any particular single amino acid or class of amino acid, although ≈30% are hydrophobic (Fig. 9A). Hydrophobic residues have been implicated as critical in various transcription activation domains, even in those that are classified on the basis of their predominant amino acids (32–34). All mutations of hydrophobic residues in this region, except one, strongly reduced activation function in yeast (Fig. 9B). The exception was a mutant domain with two changes, I298A and L300A, which actually led to increased activation activity (Fig. 9B).

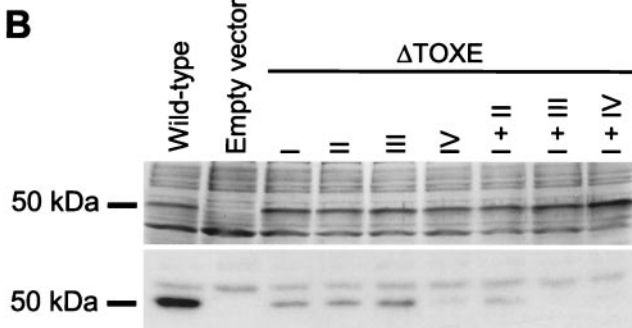
**Discussion**

TOXE was earlier shown to be required for the expression of the genes involved in the biosynthesis of HC-toxin, but the mechanism of regulation was not clear because of the unusual structure of TOXE (19). Here we show that TOXE is a DNA binding protein that recognizes a specific sequence, the tox-box, that is present in one or two copies in the promoters of all of the known genes involved in HC-toxin biosynthesis, except TOXE itself. The tox-box is functional in yeast in both orientations, which is biologically significant because two pairs of the *TOX2* genes (*HTS1/TOXA* and *TOXF/TOXG*) are tightly and divergently clustered and therefore share overlapping promoters (13, 18).

The N-terminal bZIP-type basic region is required for DNA binding. The southwestern blotting results indicate that TOXE binds as a monomer or homomultimer. Because the tox-box does not contain dyad symmetry, TOXE probably binds as a monomer. In this regard, TOXE more closely resembles transcription factors that are known to bind as monomers—e.g., Skn-1 from *Caenorhabditis elegans*—rather than most true bZIP proteins, which typically bind as homo- or heterodimers to DNA sites with dyad symmetry. Skn-1 lacks a leucine zipper but binds to an asymmetrical DNA binding site as a monomer via its bZIP-like basic region (35). However, Skn-1 differs significantly from

**A**

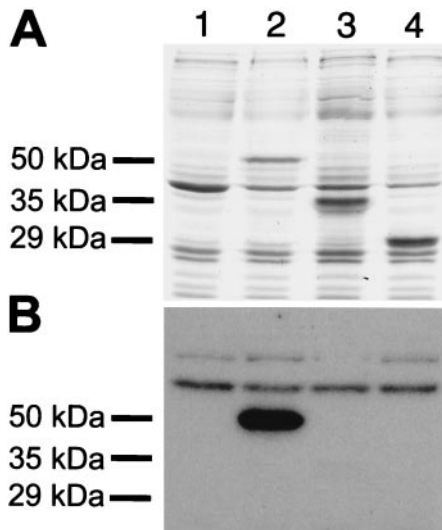
Expression construct	Basic region sequence	$\beta$ -galactosidase units
Wild-type	19 <u>RRKLQNRVAQRKYRTR</u> 34	72
Mutant I	<u>AAKLQNRVAQRKYRTR</u>	2
Mutant II	RRKLQNA <u>VAQRKYRTR</u>	3
Mutant III	RRKLQNRVAQA <u>AYRTR</u>	2
Mutant IV	RRKLQNRVAQRKY <u>ATA</u>	4
Mutant I+II	<u>AAKLQNAVAQRKYRTR</u>	3
Mutant I+III	<u>AAKLQNRVAQAAYRTR</u>	3
Mutant I+IV	<u>AAKLQNRVAQRKYATA</u>	2
Empty vector	-----	2



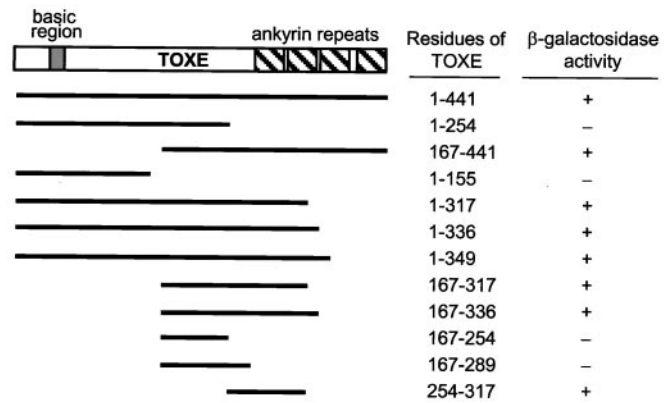
**Fig. 6.** Mutations in the TOXE basic region reduce *in vivo* transcriptional activity and *in vitro* DNA binding. (A) Underlined amino acids indicate the changes made in TOXE in each mutant.  $\beta$ -Galactosidase activities were measured in yeast strain YKP50.1 (see Fig. 5) expressing mutant TOXE constructs. (B) Southwestern blotting. Mutant TOXE constructs were expressed in *E. coli*. (Upper) The SDS/PAGE gel stained with Coomassie blue; (Lower) the autoradiograph of the blot probed with a  $^{32}\text{P}$ -labeled DNA fragment from the *TOXA/HTS1* promoter containing a single *tox*-box.

TOXE in containing homeodomain elements and lacking ankyrin repeats (35, 36).

Two versions of TOXE that were truncated at the carboxy terminus failed to bind to the *tox*-box, suggesting that the



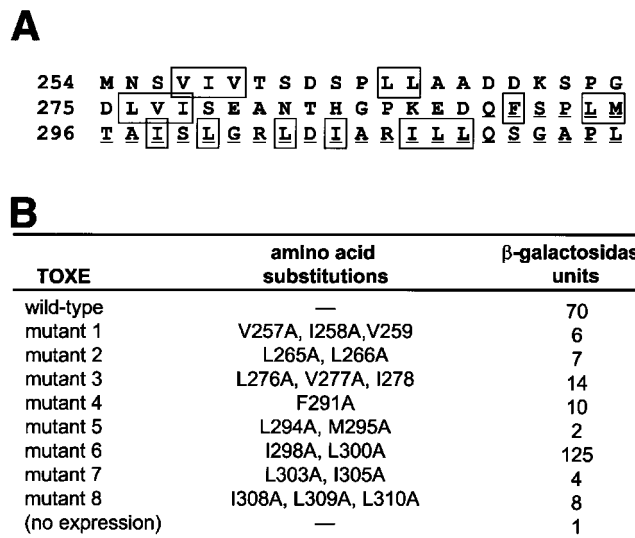
**Fig. 7.** TOXE lacking the carboxy-terminus does not bind DNA. (A) SDS/PAGE (stained with Coomassie blue) of total extracts of *E. coli* expressing different TOXE constructs. Lane 1, empty plasmid (negative control); lane 2, full-length TOXE (amino acids 1–441) (positive control); lane 3, TOXE amino acids 1–317; lane 4, TOXE amino acids 1–254. The sizes of the expressed proteins are given on the left. (B) Southwestern blotting of the gel shown in A probed with a DNA fragment from the *TOXA/HTS1* promoter containing a single *tox*-box.



**Fig. 8.** Mapping of the TOXE activation region. The indicated fragments of TOXE were fused to the  $\text{GAL4}_{\text{DBD}}$ , expressed in yeast strain Y190, and the yeast transformants assayed for  $\beta$ -galactosidase activity.

C-terminal ankyrin repeats contribute either directly or indirectly to DNA binding. The four-and-a-half N-terminal ankyrin repeats of the  $\beta$  subunit of the transcriptional regulator GABP stabilize the interaction between the  $\text{GABP}\alpha/\beta$  complex and DNA (37). Therefore it is possible that the ankyrin repeats of TOXE contribute to the stability of TOXE binding to DNA or are critical for the correct positioning of the basic region over the *tox*-box.

In addition to DNA binding activity, TOXE also has the capacity to activate transcription in a heterologous system, indicating that TOXE contains an activation domain. The six ankyrin repeats of the *C. elegans* GLP-1 receptor are alone sufficient to activate transcription in yeast (38). However, the activation domain of TOXE defined in our experiments resides in a 63-aa portion of the protein just before and including the first ankyrin repeat. All four ankyrin repeats are not necessary for TOXE to function as a transcriptional activator, although it cannot be excluded from our experiments that the four ankyrin domains might constitute a second activation domain in TOXE.



**Fig. 9.** Mutational analysis of the activation domain of TOXE. (A) Sequence of the activation domain. Hydrophobic residues that were mutated are boxed, and the first ankyrin repeat is underlined. (B) Full-length TOXE proteins containing the indicated mutations were fused to the  $\text{GAL4}_{\text{DBD}}$ , expressed in yeast strain Y190, and the yeast transformants assayed for  $\beta$ -galactosidase activity.

The only known phenotype of *TOXE* mutants is inhibition of mRNA expression of the *TOX2* genes dedicated to HC-toxin biosynthesis, with concomitant loss of specific pathogenicity of *C. carbonum* on maize that is homozygous recessive at the *Hm1* and *Hm2* loci (19). Like the other genes of *TOX2*, *TOXE* is present only in *Tox2*<sup>+</sup> isolates of *C. carbonum* isolates, and at least one of its two copies is clustered with the other *TOX2* genes (ref. 19; J.-H. Ahn and J.D.W., unpublished data). Therefore, *TOXE* appears to be a pathway-specific transcription factor whose sole function is to coordinate the expression of the genes involved in HC-toxin biosynthesis.

A gene whose product has the same structure as *TOXE*, namely a bZIP basic region, ankyrin repeats, and no leucine zipper, was recently reported in *Cladosporium fulvum*, a fungal pathogen of tomato (39). Thus, *TOXE* might be the prototype of a new class of transcription factors, so far found only in plant pathogenic filamentous fungi. Bussink *et al.* (39) have proposed the name bANK proteins for this class of transcription factor.

Pathway-specific transcription factors have also been found to regulate secondary metabolite biosynthesis in other filamentous

fungi. For example, *TRI6*, a zinc-finger transcription factor, regulates the expression of the trichothecene biosynthetic genes of *Fusarium sporotrichioides* (40, 41). In species of *Aspergillus*, the aflatoxin biosynthetic genes are regulated by *AFLR*, a zinc cluster transcription factor (42–45). Another zinc cluster protein, *ORFR*, regulates the ACR-toxin gene cluster of *Alternaria alternata* (46). In all of these cases, the transcriptional regulators are clustered with the biosynthetic genes, as is the case in *TOX2*. Because trans-acting transcription factors, such as *TOXE*, *TRI6*, *AFLR*, and *ORFR*, should be capable of activating genes anywhere in their respective genomes, the biological rationale for the physical linkage of regulatory and regulated genes in fungal secondary metabolite gene clusters is not clear. It may be a result of their evolutionary origins and mechanisms of transmission (47–49).

We thank John Pitkin for advice in the early stages of this work and Steve Triezenberg for valuable suggestions throughout. This work was supported by the U.S. Department of Energy, Division of Energy Biosciences.

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