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CHARACTERIZATION OF A TRANSCRIPTION FACTOR UNIQUE TO RACE 1 ISOLATES OF COCHLIOBOLUS CARBONUM

By

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ABSTRACT

CHARACTERIZATION OF A TRANSCRIPTION FACTOR UNIQUE TO RACE 1 ISOLATES OF COCHLIOBOLUS CARBONUM

By

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HC-toxin is a cyclic tetrapeptide produced by race 1 isolates of the filamentous fungus *Cochliobolus carbonum*. Genetic and biochemical data indicate that HC-toxin is the key determinant of virulence in interactions between toxin-producing isolates of the fungus and maize lines homozygously recessive at the *Hm* locus. Production of HC-toxin segregates as a single genetic locus, *TOX2*, in crosses between race 1 isolates and race 2 isolates that do not produce the toxin. Molecular analyses of the *TOX2* locus have revealed that at least six linked genes are necessary for HC-toxin biosynthesis. One of these genes, *TOXE*, was shown to be necessary for the full expression of most of the genes within the *TOX2* locus. In this study the product of the *TOXE* gene is shown to be a transcription factor that binds to a conserved DNA sequence present in one or more copies within the promoters of the genes that comprise the *TOX2* locus. Through mutational analysis of the TOXE protein, regions critical for proper DNA binding and transcriptional activation were identified. The biochemical properties of TOXE as well as its role in HC-toxin production and pathogenicity are discussed.

To my mom, who gives so much and asks for so little

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INTRODUCTION

Although plants are in constant contact with microorganisms in their environment, these encounters are usually innocuous, as most microbes are unable to infect plants. Even encounters with microbes that are genuine plant pathogens usually do not result in disease. Failure of a pathogen to infect and elicit disease symptoms is often due to the fact that the plant does not support the niche requirements of the potential pathogen and is therefore a non-host. Encounters between non-host plants and phytopathogens are referred to as incompatible interactions. Plants are resistant if they contain preformed chemical or structural barriers that the pathogen is unable to overcome, or if they can detect the pathogen and mount an active defense to kill or restrict the growth of the invading organism (Hammond-Kosack and Jones, 1996). Compatible interactions occur when pathogens have the ability to subvert, suppress, or overcome a host plant's natural resistance.

Phytopathogens are specialists amongst microorganisms. Microbes that can infect plants have acquired traits that allow them to occupy a specialized niche that would otherwise be restrictive. However, traits that allow a pathogen to infect one host do not necessarily allow it to infect another. While some phytopathogens are able to infect a wide range of hosts, sometimes belonging to many families of higher plants, others are restricted to a single species or even a particular accession within a species (Agrios, 1997). Likewise, the degree to which a pathogen can infect and cause disease also varies from one pathogen to another. Even within a given pathogen species there is often

variability, as some isolates of a species are virulent pathogens on host plants that other isolates of the same species are unable to infect.

The factors that allow microbes to successfully infect plants are usually classified as either general or specific pathogenicity factors. General pathogenicity factors are traits that are common to all pathogens. In contrast, specific pathogenicity factors are unique traits required for a compatible interaction between a specific pathogen and host. These can range from specific toxins that act on a particular host to enzymes that allow for the colonization of a particular tissue. Such traits are most obvious in comparisons between isolates of a pathogen that have different host ranges, and to date have contributed the most to our understanding of the biochemical and molecular factors that allow some microbes to cause disease (Yoder, 1980; Nishimura and Kohmoto, 1983; Scheffer and Livingston, 1984; Alfano and Collmer, 1996; Walton, 1996).

Fungal plant pathogens

Among the causal agents of infectious plant diseases, phytopathogenic fungi play the dominant role (Knogge, 1996). The vast majority of fungi are strict saprophytes, but many can opportunistically invade damaged or impaired plant tissue. Such fungi can infect many different plant species, and are of particular importance with regard to post-harvest spoilage. Other fungi are able to invade and colonize living tissue of healthy host plants. These fungi are responsible for significant annual crop losses and have caused several severe epidemics (Knogge, 1996).

With the possible exception of the highly evolved obligate biotrophs, most phytopathogenic fungi share many of the same characteristics as the nonpathogenic

saprophytes to whom they are closely related (Agrios, 1997). Indeed, most plant pathogenic fungi retain the capacity to survive as saprophytes in the absence of their host(s). Some of the attributes that enable these fungi to live as saprophytes probably contribute to their ability to colonize plants. However, it is the traits that do allow them to infect living plants that are of particular interest to plant pathologists.

Like other plant pathogens, variability often occurs within a fungal species, which enables some isolates to infect host plants that other isolates of the same species cannot. The specialized forms of a particular fungal species that have unique host ranges are classified as different *formae speciales*, which are often further divided into different races (Walton, 1997). Theoretically, the differences between isolates at the race level can be subtle, differing at a single gene, or quite pronounced, involving entire biochemical pathways. In either case, these differences represent specific pathogenicity factors that allow for host specificity.

While it is not completely understood how different races within a species develop, it likely involves a combination of genetic changes within both the pathogen and the host. It is easy to envision that different races of fungi can arise by genetic changes that result in either a gain or loss in function. A loss of function mutation in any gene essential for pathogenicity or virulence in a fungus capable of surviving saprophytically would result in the evolution of a new race. Similarly, gain of function events that enable a fungus to infect a broader host range or become more virulent could also lead to the establishment of new races. Many, if not most, plant pathogenic fungi probably arose as the result of gain of function changes at some point in their evolution.

Infection of the host

Once contact has been initiated between a fungal pathogen and a suitable host plant, two distinct events must transpire for the pathogen to be successful. First, the pathogen must gain entry into the host plant. The cuticle and the epidermal cell walls of plants serve as the first barriers to infectious agents of disease. Therefore the fungus must have a way to traverse the plant surface. To accomplish this, fungi have developed at least two distinct strategies. Some fungi, like *Magnaporthe grisea*, the casual agent of rice blast disease, use mechanical pressure to pierce the cell walls of their hosts (Howard *et al.*, 1991). These fungi can generate extremely high turgor pressures inside of large specialized structures, called appressoria, on the surface of the host. An extension of the appressorium, the penetration peg, is then pushed through the plant cell.

A second method employed by some fungi involves digesting the host's cell walls. Most saprophytic fungi and closely related plant pathogens secrete enzymes that can dissolve plant cell walls, enabling the fungi that produce them access into the plant. Additionally, the action of these enzymes serves to release fragments of the cell wall polymers that may also be used as a source of nutrients for the advancing mycelium.

While it is somewhat easy to envision that the first strategy mentioned may have evolved for the sake of pathogenicity, the second probably did not. Since enzymes that can digest plant cell wall polymers are also required for a saprophytic lifestyle, they probably do not represent specialized tools that have evolved directly as specific pathogenicity factors. And while having the capacity to produce an arsenal of plant cell wall degrading enzymes (CWDEs) is widely believed to contribute to pathogenicity,

strong experimental evidence (with the possible exception of cutinases) linking any particular class of CWDE to pathogenicity is lacking (Schafer, 1994).

Once a fungal pathogen has entered the plant the second stage of the disease process begins. This is the colonization of the host tissue where the fungus attempts to absorb nutrients from the host and complete its life cycle, which often involves modification of the plant tissue. During this stage the infected plant typically tries to mount an active defense response. Known responses include fortification of cell walls near the site of entry, release or production of substances toxic to the pathogen, localized cell death, activation of gene expression, and the initiation of local and systemic signals to trigger other defense responses aimed at stopping the pathogen from further colonization (Hammond-Kosack and Jones, 1996).

To combat the action of the plant many fungi secrete toxic substances or hormone-like compounds that can kill host cells or interfere with the host's metabolism and defense responses. Fungal toxins are generally low molecular weight secondary metabolites that vary with regard to specificity. Toxins with low specificity that are active on a broad range of plant species are classified as host-nonselective toxins (Walton, 1996). Nonselective phytotoxic substances like fusicoccin, trichothecene, coronatine, phaseolotoxin, syringomycin, and tabtoxin are produced by a wide variety of pathogens and are known to contribute to the development of symptoms and virulence (Stoessl, 1981; Ballio and Graniti, 1991; Proctor *et al.*, 1995; Knogge, 1996). However, by definition, these nonselective toxins are not primary determinants of host range (Walton, 1996). And although the modes of action of most nonselective toxins are poorly

understood (Knogge, 1996) it is reasonable to expect that such toxins probably affect processes shared amongst plants that are vital to their ability to maintain homeostasis.

In contrast, genetic and biochemical studies have shown that some toxins are key determinants of host range, in that resistance or susceptibility to a particular fungal pathogen always correlates with the host's sensitivity or insensitivity to the toxin produced by that pathogen (Walton, 1996). Two fungal genera, *Alternaria* and *Cochliobolus*, are particularly well known for their ability to produce host-selective toxins (HSTs), and the biosynthesis and mode of action of several toxins within these genera have been studied (Walton, 1996).

Production of HSTs and secondary metabolites

A full understanding and appreciation of the role of HSTs in pathogenicity requires an understanding of their synthesis and biochemistry. Most HSTs produced by fungi are low molecular weight secondary metabolites (Walton, 1996). Secondary metabolites have diverse structures and are defined as substances not absolutely required for normal growth and development of the organisms that produce them (Bentley, 1999). That is, if a secondary metabolite pathway is eliminated or blocked, the organism will continue to grow, at least in culture. Because secondary metabolites are not essential to the producing organism, it has been hypothesized that they likely play an ecological role (Keller and Hohn, 1997). In recent years support for this hypothesis has come from several studies implicating specific secondary metabolites in fungal-plant interactions (Panaccione *et al.*, 1992; Yang *et al.*, 1996; Desjardins and Hohn, 1997).

Despite the diverse array of secondary metabolites produced by fungi, they are all thought to be produced from a limited number of primary metabolites used in novel ways. The major pathways for secondary metabolites include the isoprenoid pathway, the polyketide pathway, the shikimate pathway, and the use of amino acids as precursors. Additionally, some compounds are derived from carbohydrates, intermediates of the tricarboxylic acid cycle, and combinations of multiple pathways (Bentley, 1999). This is reflected by some of the approximately 20 documented HSTs that can be chemically classified as polyketides, terpenoids, cyclic peptides, and saccharide derivatives (Walton, 1996).

HSTs represent only a tiny subset of the remarkable array of complex secondary metabolites produced by filamentous fungi. And since biosynthetic enzymes for only a limited number of HSTs have been identified (Walton, 1996; Yang *et al.*, 1996; Tanaka and Tsuge, 1999; Johnson *et al.*, 2000), the study of how other secondary metabolites are made has contributed to our understanding of HST biochemistry. Some well-studied examples of fungal toxins for which many biosynthetic enzymes have been identified include trichothecenes, and two structurally related compounds, aflatoxins and sterigmatocystins (Desjardins and Hohn, 1997). Analysis of the biosynthesis of these compounds illustrates several emerging themes in secondary metabolite production.

Trichothecenes are sesquiterpenoid mycotoxins produced by several genera of filamentous fungi (Keller and Hohn, 1997). They inhibit eukaryotic protein synthesis and therefore pose serious health risks if ingested. Trichothecenes cause acute and chronic mycotoxicoses in humans and farm animals that consume wheat, rye, oats, rice, and maize contaminated with *Fusarium* species that produce trichothecene toxins (Desjardins

and Hohn, 1997). They have also been implicated as virulence factors in interactions between the species that produce them and their host plants (Proctor *et al.*, 1995). Because of the worldwide interest in trichothecene contamination, the chemistry, genetics, and toxicology of these compounds have been well studied (Desjardins and Hohn, 1997).

Three structurally related compounds, T-2 toxin, diacetoxyscirpenol (DAS), and deoxynivalenol (DON) are the trichothecenes most commonly found in agricultural commodities infected with *Fusarium* species. These three compounds, and all trichothecenes, share a tricylic core structure called trichothecene. Trichothecene is produced from the precursor trichodiene through an ordered series of oxygenations, isomerizations, and esterifications (Desjardins *et al.*, 1993). To date, 10 closely linked genes involved in trichothene production have been characterized in *F. sporotrichioides*, which produces both T-2 toxin and DAS (Keller and Hohn, 1997). Mutational analysis of five of these genes, *TRI3*, *TRI4*, *TRI5*, *TRI6*, and *TRI11*, have confirmed their involvement in toxin production (McCormick *et al.*, 1996; Hohn *et al.*, 1995; Hohn and Desjardins, 1992; Alexander *et al.*, 1998; Hohn *et al.*, 1999). Furthermore, accumulation of intermediates in the mutant strains has provided evidence for the proposed biosynthetic pathway (Desjardins *et al.*,1993).

The genes required for trichothecene biosynthesis in *F. sporotrichioides* appear to be clustered (Keller and Hohn, 1997). Gene clusters are defined as two or more genes involved in the same pathway that lie adjacent to one another (Keller and Hohn, 1997). One of the genes within this cluster, *TRI6*, appears to be required for the expression of the other genes that comprise the cluster (Proctor *et al.*, 1995). The *TRI6* gene product

contains three copies of the Cys_2His_2 zinc-finger motif at its C-terminus and has been shown to be a pathway-specific transcription factor (Hohn *et al.*, 1999).

Aflatoxins (AFs) and sterigmatocystin (ST) are among the most highly toxic, mutagenic, and carcinogenic natural products known (Brown *et al.*, 1996). AFs and ST together comprise a group of closely related polyketide mycotoxins derived from the same biochemical pathway, with ST representing the penultimate precursor of AF (Bennett and Papa, 1988; Brown *et al.*, 1996). The shared biochemical pathway leading to the biosynthesis of AFs and ST represents one of the best studied pathways of fungal secondary metabolites (Brown *et al.*, 1999). AFs are produced by certain strains of *Aspergillus parasiticus*, *A. flavus*, *A. nomius*, and *A. tamarii*, and ST is produced by several ascomycetes and deuteromycetes including *A. nidulans*.

Most of what is known about the genes required for the biosynthesis of AFs and ST comes from work performed with *A. flavus*, *A. parasiticus*, and *A. nidulans*. In *A. nidulans*, twenty-five co-regulated genes comprise a gene cluster required for ST production (Brown *et al.*, 1996). Not surprisingly, many of the genes required for ST biosynthesis by *A. nidulans* have close homologs in AF producing species. One gene in particular, *alfR*, found in the ST gene cluster of *A. nidulans*, has close homologs in the AF gene clusters of *A. flavus* and *A. parasiticus* (Yu *et al.*, 1996). The *alfR* gene product is a member of the zinc binuclear family of transcription factors (Todd and Andrianopoulos, 1997) typified by the *Saccharomyces cerevisiae* Gal4 DNA binding protein (Giniger *et al.*, 1985). AflR is required for the expression of the genes of both the AF and ST gene clusters (Yu *et al.*, 1996).

Through the molecular analysis of many fungi that produce secondary metabolites, it is now clear that secondary metabolite production by filamentous fungi frequently involves the expression of a set of genes that are closely linked into gene clusters. Comparison of secondary metabolism gene clusters from several fungi has revealed that most are comprised of genes encoding enzymes involved in the synthesis of the product as well as transcription factors that coordinate the expression of the cluster (Keller and Hohn, 1997). In addition to the clusters identified for trichothecene and AF/ST production, examples of secondary metabolite clusters in fungi include those for penicillin (Smith *et al.*, 1990), gibberellins (Tudzynski and Holter, 1998), ergot alkaloids (Tudzynski *et al.*, 1999), AK-toxin (Tanaka and Tsuge, 1999), and HC-toxin (Ahn and Walton, 1999). In addition, there is now evidence for a gene cluster involved in fumonisin biosynthesis (Proctor *et al.*, 1999) and it is likely that there is a gene cluster involved in AM-toxin production (Johnson *et al.*, 2000).

Northern leaf spot and ear mold disease of maize

Cochliobolus carbonum R. R. Nelson (anamorph, *Bipolaris zeicola* (G. L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) is the causal agent of Northern leaf spot and ear mold disease of maize (*Zea mays* L.). The disease was first noticed in 1938 on the dent corn inbred line "Pr" (Ullstrup, 1941), which was bred from an open-pollinated cultivar, Proudfit Reid (Gerdes *et al.*, 1993). The pathogen was isolated and found to be a fungus that closely resembled *Helminthosporium maydis*. All isolates were morphologically similar, but two groups were distinguishable based on the symptoms they produced and on the lines of corn they infected. One group was highly virulent

giving rise to well-defined, zonate, rapidly spreading lesions on the foliage and pronounced black mycelium on the kernels of infected ears. The other group was weakly pathogenic, unable to colonize much beyond the site of penetration and, therefore, causing only mild chlorotic-necrotic flecks on the leaves. The highly virulent and weakly virulent isolates were classified as variant forms of *H. maydis* and divided into two physiological races, race 1 and race 2, respectively (Ullstrup, 1941). Further characterization of the pathogen revealed that it was not *H. maydis*, but a new species that was renamed *H. carbonum*, reflecting the charred appearance of the infected ears (Ullstrup, 1944). In 1959 the sexual stage of *H. carbonum* was discovered, which indicated that the pathogen was an ascomycete belonging to the genus *Cochliobolus* (Nelson, 1959).

Genetic crosses between race 1 and race 2 isolates of *C. carbonum* revealed that virulence is determined by a single genetic locus, *Tox2*, which also confers the ability to produce the secondary metabolite *H. carbonum* (HC)-toxin (Scheffer and Ullstrup, 1965; Scheffer *et al.*, 1967). The major form of HC-toxin (HC-toxin I) is a cyclic tetrapeptide with the structure cyclo (D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid (Walton *et al.*, 1982). Three minor forms (HC-toxins II, III, IV) differ slightly in amino acid composition and each varies with respect to biological activity from HC-toxin I (Kim *et al.*, 1985; Tanis *et al.*, 1986; Rasmussen, 1987; Rasmussen and Scheffer, 1988; Liesch *et al.*, 1982; Pope *et al.* 1983; Gross *et al.*, 1982). The major form of HC-toxin and all of its naturally-occurring analogs require both the terminal epoxide and the 8-carbonyl group of Aeo for biological activity (Walton and Earle, 1983, Ciufetti *et al.*, 1983, Kim *et al.*, 1987).

C. carbonum race 1 is one of the most aggressive and severe pathogens that attacks maize; however, most maize germplasm is resistant. A single dominant gene, *Hm1*, governs resistance and confers complete protection at all stages of growth (Ullstrup, 1941; Nelson and Ullstrup, 1964). Using a cell-free extract from a resistant maize genotype (*Hm1/hm1*) Meeley and Walton (1991) were able to show that an NADPH-dependent enzyme that reduced the 8-carbonyl group on Aeo inactivated HC-toxin. Subsequently, this HC-toxin reductase activity (HCTR) was shown to be present in all maize extracts from resistant genotypes (*Hm1/hm1, Hm1/Hm1*) tested, but absent in susceptible ones (*hm1/hm1*) (Meeley *et al.*, 1992). *Hm1* was cloned by Johal and Briggs (1992) and its predicted product was shown to be similar to known NADPH-dependent reductases. Furthermore, the cloning of *Hm1* revealed that HCTR activity alone is sufficient to prevent severe infection by *C. carbonum* race 1 (Meeley *et al.*, 1992; Johal and Briggs, 1992). HCTR activity is detectable in extracts of several other grasses (*e.g.*, barley, oats, and wheat) and therefore may represent an ancient resistance strategy within the *Poaceae* against HC-toxin and similar compounds (Meeley and Walton, 1993; Multani *et al.*, 1988). HCTR activity has not been found in dicotyledonous plants, and the basis of the insensitivity of dicotyledonous plants to HC-toxin is not known (Meeley and Walton, 1993).

A second maize gene for resistance to *C. carbonum* race 1, *Hm2*, has been shown to be a duplicate of *Hm1* (Multani *et al.*, 1998). However, *Hm2* differs from *Hm1* by providing only partial resistance to mature plants (Multani *et al.*, 1998). All maize inbreds that are completely susceptible to *C. carbonum* race 1, including Pr, are homozygous recessive at both *hm1* and *hm2* (Multani *et al.*, 1998).

It is not known how HC-toxin enables *C. carbonum* to colonize the tissue of susceptible maize (Walton, 1996). HC-toxin is different from most of the known phytotoxins in that it appears to be cytostatic rather than cytotoxic (Walton and Panaccione, 1993). Therefore, the mode of action seems to be more subtle than just killing host cells in advance of the growing mycelium (Walton and Panaccione, 1993). Studies in which homozygously recessive (*hm1/hm1*) maize plants were inoculated with race 2 spores prior to inoculation with the normally virulent race 1 spores resulted in typical race 2 disease symptoms (Cantone and Dunkle, 1990). This study hints at the possibility that HC-toxin suppresses an active defense response mounted by the host plant. Thus, HC-toxin may act to perturb signal transduction or gene regulation within the host cell.

The hypothesis that HC-toxin may have a negative influence on gene expression in maize is supported by experiments testing the effects of HC-toxin and the related cyclic peptide chlamydocin (cyclo[α-aminobutyric acid-L-Phe-D-Pro-L-Aeo]) on maize histone deacetylase (HDAC) activity (Brosch *et al.*, 1995). Brosch *et al.* (1995) showed that both of these Aeo-containing cyclic peptides inhibited maize HDAC activity *in vitro*, and this was later confirmed by *in vivo* data by Ransom and Walton (1997). Both of these studies are in agreement with the report that another Aeo-containing cyclic peptide, trapoxin (cyclo[L-Phe-L-Phe-D-Pip-L-Aeo]), inhibits mammalian HDAC activity *in vivo* and *in vitro* (Kijima *et al.*, 1993). These findings are of significance given that histone acetylation is now recognized as one of the major strategies by which eukaryotes remodel chromatin and hence regulate gene expression (Sterner and Berger, 2000; Cheung *et al.*, 2000). What genes are affected in maize by the state of histone acetylation in response to

the presence of HC-toxin and how this may leads to a compatible interaction is not known (Walton, 1996).

Biosynthesis of HC-toxin

Molecular analysis of the *TOX2* locus has led to the discovery of some of the genes required by race 1 isolates of *C. carbonum* to produce HC-toxin. The genes of the *TOX2* locus are loosely clustered within ~540 kb of DNA and are present only in race 1 isolates (Ahn and Walton, 1996). These genes can be classified as having biosynthetic, secretory, or regulatory functions based on sequence comparisons to known genes as well as biochemical and genetic data.

Biosynthesis of HC-toxin involves the activity of a cyclic peptide synthetase, HTS (Walton, 1987; Walton and Holden, 1988). HTS is the product of the gene *HTS1*, which is composed of a single 15.7-kb open reading frame (Scott-Craig *et al.*, 1992). *HTS1* is found in two copies in most race 1 isolates of *C. carbonum* (Ahn and Walton, 1996). When both copies of *HTS1* are simultaneously inactivated using targeted gene disruption, HC-toxin production is lost and the transformed strain is only weakly pathogenic (Panaccione *et al.*, 1992).

Hybridization analysis showed that *HTS1* is part of a larger contiguous region of 22 kb of DNA that is unique to race 1 isolates of *C. carbonum*. Sequence analysis of the DNA flanking the 5' end of *HTS1* revealed the presence of another gene, *TOXA*, that is also unique to HC-toxin producing isolates (Pitkin *et al.*, 1996). The predicted product of *TOXA* exhibits a high degree of similarity to small molecule efflux pumps and is thought to be involved in the secretion HC-toxin (Pitkin *et al.*, 1996). An HC-toxin efflux pump

may be important for delivering HC-toxin to the host during the infection process and/or protecting the fungus from the toxic effects of HC-toxin (Pitkin *et al.*, 1996). Supporting the argument that the TOXA protein is essential for protection of the fungus from its own toxin, Pitkin *et al.* (1996) were unable to recover any mutants with disruptions in both copies of *TOXA*.

In addition to *HTS1* and *TOXA*, three other genes (*TOXC*, *TOXG*, and *TOXF*) unique to race 1 isolates of *C. carbonum* have been cloned and shown to be essential for the biosynthesis of HC-toxin. *TOXC* and *TOXF* are both thought to be involved in the synthesis of Aeo (Ahn and Walton, 1997; Cheng *et al.*, 1999). The predicted product of *TOXC* is highly similar to the β-subunit of fatty acid synthases from several lower eukaryotes, and contains domains predicted to encode acetyl transferase, enoyl reductase, dehydratase, and malonyl-palmityl transferase (Ahn and Walton, 1997). *TOXF* is predicted to encode a protein with moderate homology to many known or putative branched-chain-amino-acid transaminases (Cheng *et al.*, 1999). *TOXC* and *TOXF* have been analyzed by targeted gene disruption and both have been found to be essential for HC-toxin production and virulence (Ahn and Walton, 1997; Cheng *et al.*, 1999). Predictions based on sequence analysis of *TOXG*, as well as biochemical and genetic evidence, reveal that it encodes an alanine racemase (Cheng and Walton, 2000). Analysis of strains in which all copies of *TOXG* have been disrupted demonstrates that the TOXG protein is necessary to produce the three forms of HC-toxin that contain D-Ala (HC-toxins I, III, IV). However, *TOXG* null strains are still able to produce HC-toxin II and are nearly as virulent as wild-type race 1 strains (Cheng and Walton, 2000). A fourth gene, *TOXD*, has also been found within the *TOX2* locus; however, null mutants for *toxD*

have no noticeable effect on HC-toxin production (Y. Q. Cheng, and J. D. Walton, unpublished data).

Regulation of the *TOX2* locus appears to be, at least in part, under the control of another *TOX2* specific gene, *TOXE*. Deletion of *TOXE* within a strain containing a single copy resulted in the loss of HC-toxin production and reduced virulence on susceptible host plants. Furthermore, transcripts of three genes unique to *TOX2*; *TOXA*, *TOXC* and *TOXD*, are down-regulated in a *TOXE* mutant (Ahn and Walton, 1998). Two recently cloned genes, *TOXG* and *TOXF*, also require *TOXE* for expression (Cheng *et al.*, 1999; Cheng and Walton, 2000). However, despite the clear phenotype displayed in the *toxE* null strain the precise role of TOXE and how it may control HC-toxin production was not certain. This was due in part to the fact that TOXE, although it contained two structural motifs that are commonly found in transcription factors, was unlike any known transcription factor. Also, aside from the Northern blot data, no biochemical data were generated to show how TOXE may function (Ahn and Walton, 1998).

This study was undertaken to elucidate the functional properties of TOXE and to elucidate its role in HC-toxin production in *C. carbonum*. *In vitro* DNA binding indicates that TOXE specifically recognizes and binds to a short sequence found within the promoter of each *TOX2* gene that it regulates. TOXE expression in yeast also provided *in vivo* data that TOXE functions as a transcription factor with the ability to bind DNA in a sequence specific manner and has the ability to activate transcription. Finally, two site-specific mutagenesis studies established critical residues and domains within TOXE and showed how they contribute to its function.

Chapter 1

ANALYSIS OF TOXE AS A SITE-SPECIFIC DNA BINDING PROTEIN

Abstract

Race 1 isolates of the filamentous fungus *Cochliobolus carbonum* are characterized by their ability to produce HC-toxin. Production of this toxin is under the control of a single genetic locus, *TOX2*, that is unique to toxin producing strains. The predicted product of the *TOXE* gene, TOXE, has been shown to be required for the transcription of other genes within the *TOX2* locus and is thought to play a role in the regulation of HC-toxin. The data presented here indicate that TOXE functions as a site-specific DNA-binding protein that recognizes a short conserved sequence, called the tox-box, located within the promoter of each gene that it regulates. Expression of TOXE in a yeast reporter strain also shows that TOXE is capable of binding DNA *in vivo*, and is capable of activating transcription.

Introduction

Microorganisms are well known for their capacity to produce secondary metabolites such as mycotoxins, antibiotics, and pigments. In many cases, in both prokaryotic and eukaryotic organisms the genes that encode enzymes for secondary metabolite biosynthesis are clustered (Brown *et al.* 1996; Keller and Hohn 1997). This has both facilitated their study and revealed some commonalties between diverse biosynthetic pathways. One feature that seems to be common to gene clusters dedicated to secondary metabolite production is that in addition to genes encoding biosynthetic enzymes and efflux pumps, these clusters often contain regulatory genes that encode transcription factors that function to regulate the expression of the other genes within the cluster. For example, *Tri6*, which encodes a protein of the zinc-finger class of transcription factors, regulates the expression of the trichothecene biosynthetic genes of *Fusarium sporotrichioides* (Proctor *et al.* 1996; Hohn *et al.* 1999). Likewise the sterigmatocystin and aflatoxin biosynthetic gene clusters of *Aspergillus* species include genes that encode transcription factors of the zinc binuclear cluster class (Fernandes *et al.* 1998; Payne *et al.* 1993; Yu *et al.* 1996).

Race 1 isolates of the filamentous ascomycete *Cochliobolus carbonum* produce a secondary metabolite called HC-toxin. Production of HC-toxin by race 1 isolates enables them to be especially virulent on maize lines that are homozygous for the recessive allele of *Hm*. Strains of the fungus that do not produce the toxin are only weakly pathogenic towards maize, regardless of the host's genotype. Thus, HC-toxin is the critical determinant of host range, and is therefore classified as a host-selective toxin (Walton, 1996).

The biosynthesis of HC-toxin is controlled by the genetic locus *TOX2*. The *TOX2* locus is composed of at least seven genes (*HTS1*, *TOXA*, *TOXC*, *TOXD*, *TOXE*, *TOXG*, and *TOXF*), which are present in multiple copies in most laboratory strains that produce the toxin ($Tox2^+$ strains) (Ahn and Walton, 1997; Ahn and Walton 1998; Cheng *et al.* 1999; Cheng and Walton 2000; Panaccione *et al.* 1992; Pitkin *et al.* 1996; Scott-Craig *et al.* 1992). With the exception of *TOXD*, all of the known genes within the locus are required for production of HC-toxin.

One of the genes within the cluster, *TOXE*, was shown to be required for the expression of *TOXA*, *TOXC*, and *TOXD* (Ahn and Walton 1998). Subsequent analyses of *TOXG* and *TOXF* have shown that they are also regulated by *TOXE*, as transcripts for *TOXF* and *TOXG* do not accumulate in strains that lack a functional copy of *TOXE* (Cheng *et al.* 1999; Cheng and Walton 2000). These data indicate *TOXE* encodes a primary regulator of the *TOX2* locus.

Although it is likely that TOXE functions as a pathway-specific regulator of the *TOX2* locus, its precise mechanism of action was not known. This was largely due to the fact that TOXE does not belong to any known family of transcription factor, nor was it found to have any homologs in the public database (Ahn and Walton, 1998). However, TOXE does have two predicted structural motifs that are commonly found in transcription factors. TOXE contains a short sequence of basic amino acids near its N-terminus that match the consensus sequence characteristic of the basic leucine zipper (bZIP) family of DNA-binding transcriptional regulators. However, unlike the members of the bZIP family, TOXE does not contain a leucine zipper, a feature essential to the function of bZIP proteins.

TOXE is also predicted to contain four ankyrin repeats, located at the C-terminal end of the protein. Proteins containing ankyrin repeats are found in all eukaryotes, and are known to be involved in mediating protein-protein interactions (Bork, 1993). Currently the mere presence of ankyrin repeats in otherwise uncharacterized proteins is interpreted as an indicator of similar function (Sedgwick and Smerdon, 1999). Thus, it is likely that TOXE also interacts with other proteins.

This study was initiated to determine the precise role of TOXE in the production of HC-toxin. Specifically, I designed a series of experiments to assess whether TOXE functioned as a sequence-specific DNA-binding protein and to determine if TOXE had the capacity to function as a positive activator of transcription *in vivo*. Here I report the DNA binding activity of TOXE, the specific DNA sequence to which TOXE binds, and the ability of TOXE to activate transcription in yeast. These results provide the first biochemical evidence that TOXE functions as a transcription factor and provides the framework for a more detailed analysis of the DNA-binding and trans-activating mechanisms used by TOXE to regulate the transcription of genes within the *TOX2* locus.

Results

For expression in *E. coli*, the entire *TOXE* open reading frame was cloned into the plasmid pQE30 to create the TOXE expression plasmid pKP10. Stable expression of TOXE using *E. coli* M15 cells containing pKP10 was obtained by inducing actively growing cells with 0.1 mM ml^{-1} IPTG as described (see Materials and Methods). Expression of TOXE was determined by denaturing polyacrylamide gel electrophoresis comparing total protein extracts from cells containing pKP10 or the control plasmid

pQE30 containing no insert (Figure 1). Cells containing pKP10 accumulated a 49-kDa protein, the predicted size of TOXE. Subsequent analysis of the recombinant protein indicated that it was restricted to the insoluble fraction under the expression conditions used (data not shown).

For DNA-binding analysis, total protein extracts from *E. coil* cells expressing TOXE were subjected to southwestern blot analysis. Nitrocellulose blots containing the renatured protein extracts separated by SDS-PAGE were probed with ^{32}P-labeled DNA fragments representing the *TOXA/HTS1* intergenic region (Figure 2A). This region was chosen because *TOXA* was known to be regulated by TOXE, and it seemed likely that if TOXE bound to a regulatory element in the *TOXA* promoter, this binding site would probably lie somewhere between the transcriptional start sites of the *HTS1* and *TOXA* genes, which are only 386 bp apart (Pitkin *et al.*, 1996).

Analysis of the blots by autoradiography (Figure 2B) showed that one of the three DNA fragments bound to a protein of approximately 49 kDa found only in the cells containing the TOXE expression vector, and was therefore likely TOXE. This was the first direct evidence that TOXE could function as a DNA binding protein. It also indicated that the observed binding was likely sequence-specific since the immobilized protein bound strongly to only one of the three DNA fragments. Furthermore, since the binding was observed using TOXE produced in a heterologous system, it also established that TOXE did not require any other *C. carbonum* proteins to bind DNA, and therefore must bind either as a monomer or a homomultimer.

To determine if TOXE was indeed binding DNA in a sequence-specific manner,

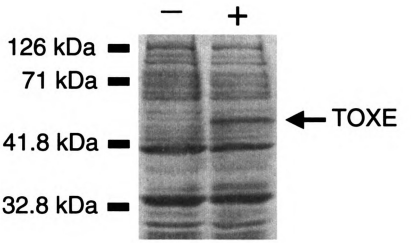


Figure 1. SDS-PAGE of total *E. coli* protein extracts after induction with IPTG. Lane 1, total protein extract from *E. coli* M15 cells containing the empty expression plasmid pQE30 (−); lane 2, total protein extract from *E. coli* M15 cells expressing TOXE from the expression vector pKP10 (+).

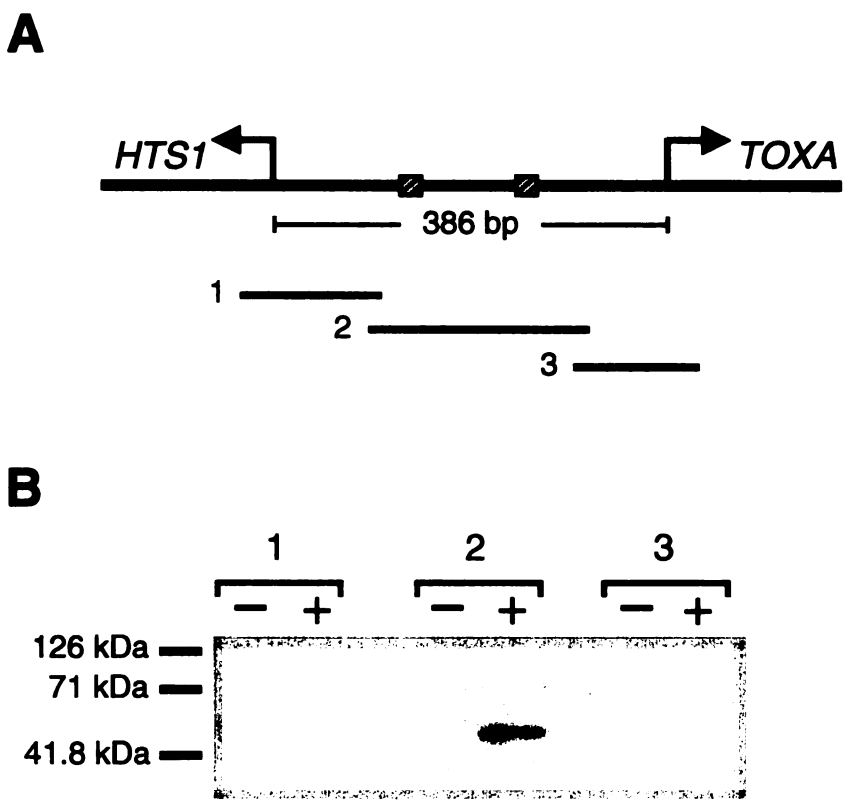


Figure 2. Southwestern blot analysis using fragments from the intergenic region between *TOXA* and *HTS1* as probes. **(A)** The intergenic region between *TOXA* and *HTS1*. The arrows indicate the transcriptional start sites of the two genes, which are 386 bp apart. Numbered lines (1-3) indicate the region of the promoter represented by each probe. Hatched boxes indicate the positions of two putative binding sites identified through subsequent analysis. **(B)** Nitrocellulose blots of *E. coli* cells either expressing (+) or not expressing (−) TOXE. Numbers above each set of lanes indicates the probe used for analysis.

a series of southwestern blot experiments were carried out using DNA fragments representing the promoters of other *TOX2* genes. The promoters of the *TOXF* and *TOXG* genes were analyzed first, because, like *HTS1* and *TOXA*, *TOXF* and *TOXG* lie adjacent to each other and are divergently transcribed from a shared intergenic region. Analysis with DNA fragments representing this region again revealed that TOXE was binding strongly to some unknown sequence present on two of the tested DNA fragments (Figure 3). Weak binding by fragments 1 and 4 was not taken to be significant (Figure 3B).

DNA sequence comparisons of the DNA fragments that were bound by TOXE in the southwestern blot assays revealed the presence of a short DNA sequence that was common to both the *TOXA/HTS1* promoter and the *TOXF/TOXG* promoter. Two copies of this putative binding site, with the sequence 5'-ATCTCACGTA-3', were present between *HTS1* and *TOXA* and also between *TOXF* and *TOXG*. Significantly, this putative binding site was present only on the fragments that were bound by TOXE, and not on those fragments that failed to bind.

Further analysis revealed that two copies of a similar sequence were also present in the *TOXC* promoter and a single copy was present in the promoter of *TOXD* (Figure 4). Using this information, primers for PCR were designed to amplify specific fragments of both the *TOX*C and *TOXD* promoters. Some of the fragments generated contained the putative binding site, while it was intentionally omitted form others. These fragments were then used as probes in two additional southwestern blot experiments (Figures 5 and 6). In agreement with the data collected from the first two southwestern blots, only those

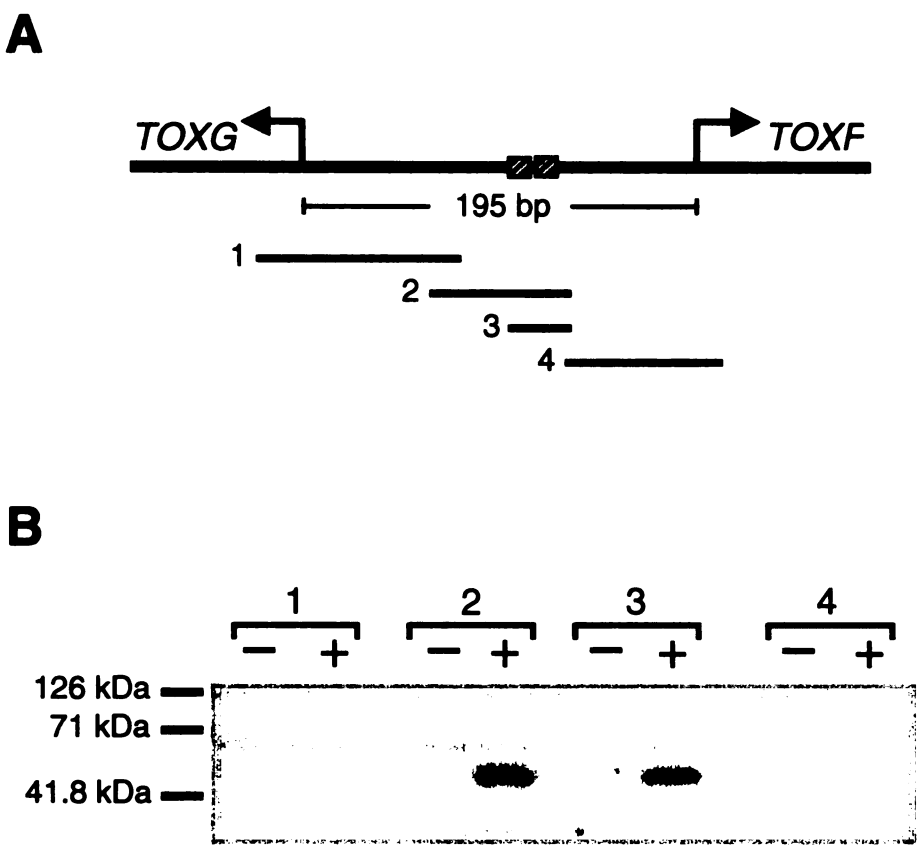


Figure 3. Southwestern blot analysis using fragments from the intergenic region between *TOXF* and *TOXG* as probes. **(A)** The intergenic region between *TOXF* and *TOXG*. The arrows indicate the transcriptional start sites of the two genes, which are 195 bp apart. Numbered lines (1-4) indicate the region of the promoter represented by each probe. Hatched boxes indicate the positions of two putative binding sites identified through subsequent analysis. **(B)** Nitrocellulose blots of *E. coli* cells either expressing (+) or not expressing (−) TOXE. Numbers above each set of lanes indicates the probe used for analysis.

Promoter	Putative binding site
TOXA/HTS1	ATCTCACGTA
TOXA/HTS1	ATCTCACGCA
TOXF/TOXG	ATCTTACGTA
TOXF/TOXG	ATCTCGCGTA
TOXC	ATCTCTCGAA
TOXC	ATCTCTCGTC
TOXD	ATCTCTAGGC
"tox-box" consensus	**ATCTCNCGNA**

Figure 4. Comparison of the putative TOXE binding sites. Sequence analysis of the promoters of the *TOX2* genes revealed the presence of a short, 10-bp sequence common to all of the genes. This sequence is found exclusively on the DNA fragments bound by TOXE in the southwestern blots performed using the promoters of *TOXA/HTS1* and *TOXF/TOXG*.

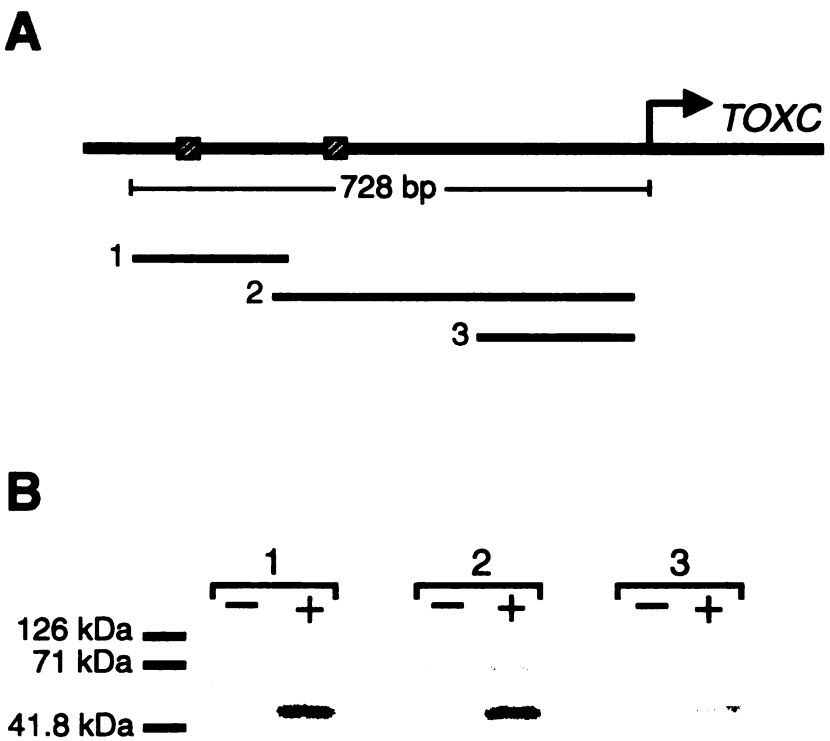


Figure 5. Southwestern blot analysis using fragments from the promoter region of *TOXC* as probes. **(A)** The promoter region of *TOXC*. The arrow indicates the transcriptional start sites of the gene which is 170 bp upstream of the start codon. Numbered lines (1-3) indicate the region of the promoter represented by each probe. Hatched boxes indicate the positions of two putative binding sites identified through subsequent analysis. **(B)** Nitrocellulose blots of *E. coli* cells either expressing (+) or not expressing (–) TOXE. Numbers above each set of lanes indicates the probe used for analysis.

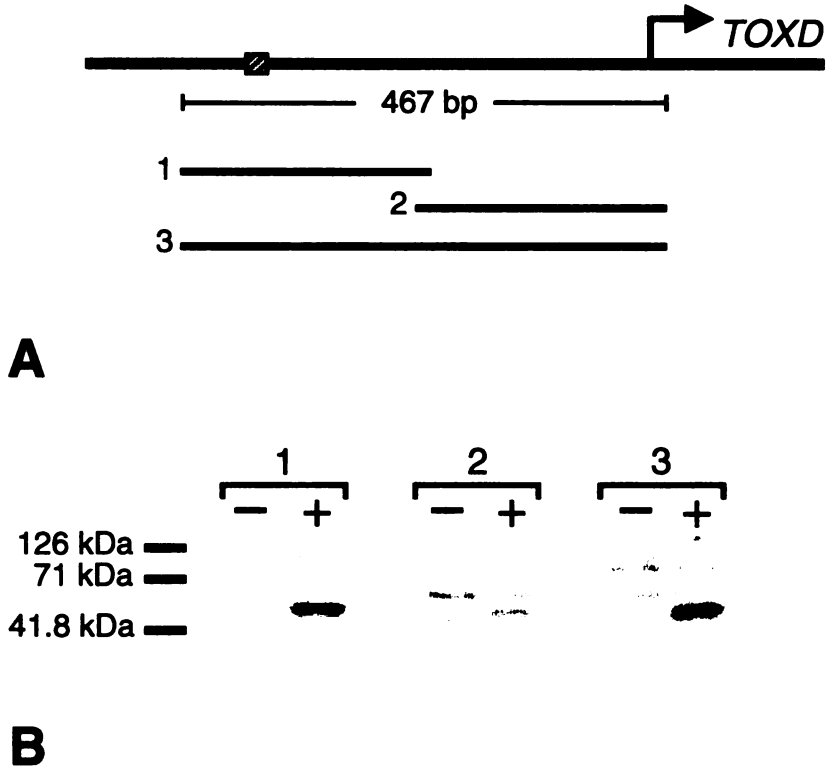


Figure 6. Southwestern blot analysis using fragments from the promoter region of *TOXD* as probes. **(A)** The promoter region of *TOXD*. The arrow indicates the transcriptional start sites of the gene which is 17 bp upstream of the start codon. Numbered lines (1-3) indicate the region of the promoter represented by each probe. Hatched box indicates the position of a single binding site identified through subsequent analysis. **(B)** Nitrocellulose blots of *E. coli* cells either expressing (+) or not expressing (–) TOXE. Numbers above each set of lanes indicates the probe used for analysis.

fragments containing the putative binding site, and not those lacking it, were bound strongly by TOXE.

Sequence comparisons of the putative binding site present in the promoters of *TOXC*, *TOXD* and the shared promoters of *TOXA/HTS1* and *TOXF/TOXG* revealed the consensus sequence 5'-ATCTCNCGNA-3' (Figure 4). For convenience, I refer to the putative binding site as the "tox-box".

To further test that the tox-box binding site identified by analysis of the promoter fragments was indeed involved in the observed binding, another southwestern blot experiment was performed, this time using synthetic, complementary oligonucleotides representing one of the tox-boxes from the *TOXA/HTS1* promoter as the probe. As a control, a mutant version of this sequence, changing the 5'-ATCTC-3' core sequence to 5'-GCAGG-3', was also used. Only the probe with the wild-type core sequence was found to bind to TOXE (Figure 7).

Based on earlier observations it was clear that TOXE could serve as a potent activator of transcription in yeast when fused to the Gal4 DNA binding domain (see chapter 3). Therefore, in light of the fact that TOXE could also bind DNA *in vitro*, it seemed likely that TOXE would be able to function independently as a transcription factor in yeast. To test this hypothesis a *lacZ* gene fused to a *Gal1* promoter lacking an upstream activation sequence was used to create a reporter gene. A DNA fragment containing a tox-box with the sequence 5'-ATCTCACGTA-3' was cloned into the *Gal1* promoter in both the forward and reverse directions. To serve as a negative control a similar construct was made using the sequence 5'-GCAGGACGTA-3', where the wild-type type 5'-ATCTC-3' core sequence had been changed. To increase the sensitivity

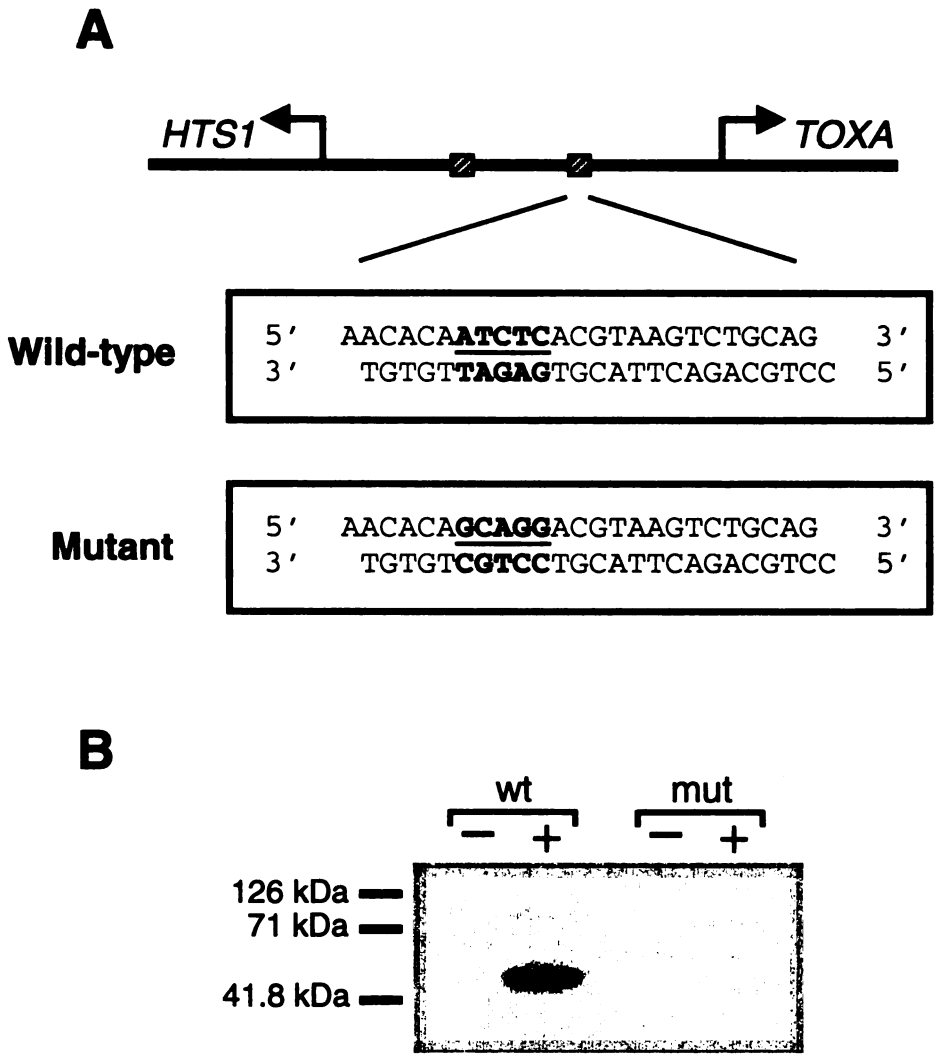


Figure 7. Southwestern blot analysis using complementary oligonucleotides as probes. **(A)** Diagram illustrating the shared promoter region between *TOXA* and *HTS1*. The relative positions of the two putative "tox-boxes" are shown as hatched boxes. The sequences of the complementary oligonucleotides used as probes representing the tox-box closest to *TOXA*, labeled wild-type, are shown. Oligonucleotides representing a mutated version of the tox-box are labeled as mutant. **(B)** Nitrocellulose blots of *E. coli* cells either expressing (+) or not expressing (–) TOXE. The lanes were probed with either the wild-type or mutant sets of oligonucleotides end labeled with ^{32}P as indicated.

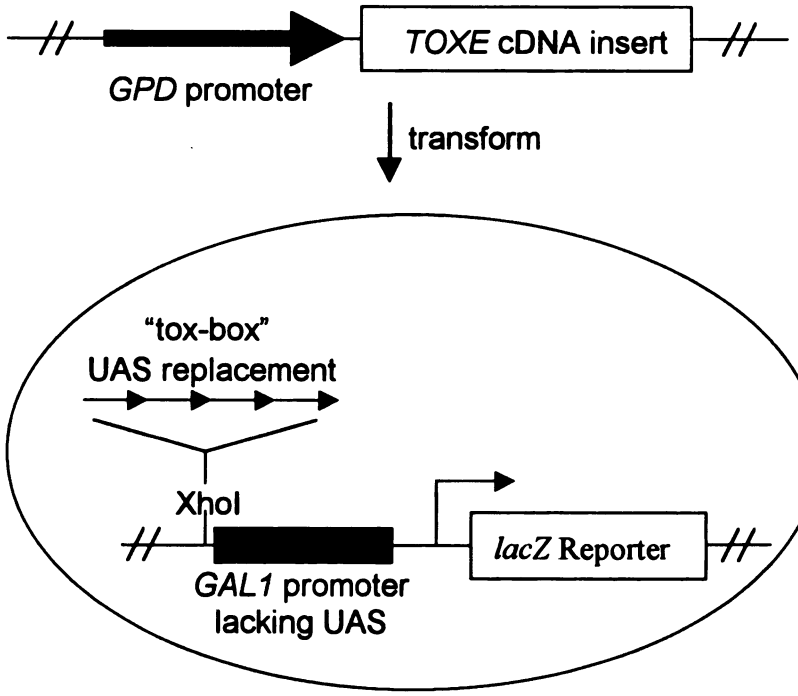
of the assay both the wild-type and the mutant versions of the tox-box sequence were constructed as 4X tandem repeats. These constructs were then integrated into the genome of the yeast strain MG109 at the *URA3* locus. A construct containing *TOXE* driven by a constitutive promoter was then cloned into each of the four reporter strains. As a negative control these strains were also transformed with the pG-1 expression vector which does not contain any insert (Figure 8A). These eight strains were then assayed for β-galactosidase activity (Figure 8B). Only the yeast strains containing the wild-type reporter construct and that were expressing TOXE had detectable levels of β-galactosidase activity. This established that TOXE could bind to DNA *in vivo* and was also able to activate transcription in yeast.

Discussion

To understand how TOXE acts to regulate the expression of the genes involved in HC-toxin biosynthesis, I tested the hypothesis that TOXE functions as a transcription factor with the capacity to bind DNA. Specifically, I tested TOXE expressed in *E. coli* to see if it was capable of binding DNA *in vitro*. The results are consistent with my hypothesis, and indicate that TOXE specifically recognizes and binds to DNA with a consensus sequence, the "tox-box", defined as 5'-ATCTCNCGNA-3'.

The analysis of TOXE in this study was largely based upon the presence of a bZIP-like basic sequence located near the amino terminus of the protein. The bZIP family of DNA-binding proteins represents one of the best studied families of transcription factors (Ellenberger, 1994). All members of the bZIP family of proteins contain two distinct, functional regions that contribute to DNA-binding. The bZIP motif

A



B

UAS replacement sequence			TOXE expression	β-galactosidase units
Strain #	tox-box	Orientation		
YKP50.1-E	Wild-type	Forward	yes	38
YKP50.3-E	Wild-type	Reverse	yes	69
YKP49.13-E	mutant	Forward	yes	0
YKP49.14-E	mutant	Reverse	yes	0
YKP50.1-pG-1	Wild-type	Forward	no	0
YKP50.3-pG-1	Wild-type	Reverse	no	0
YKP49.13-pG-	mutant	Forward	no	0
YKP49.14-pG-1	mutant	Reverse	no	1

Figure 8. Analysis of TOXE in yeast. **(A)** Schematic diagram illustrating the approach used (see text for details). **(B)** List of yeast strains used including the orientation of the tox-box sequence used to create an UAS, and the presence or absence of TOXE. β-galactosidase units (defined in methods section) for each strain are indicated.

is composed of (1) a helical segment that contains leucine residues at every seventh position, which constitutes the "leucine zipper", and (2) an associated segment that consists of 15-20 residues rich in basic amino acids, termed the basic region (Ellenberger, 1994). The leucine zippers mediate dimerization and the basic regions dictate DNA binding specificity (Ellenberger, 1994).

The bZIP family of transcription factor typically recognize DNA sites whose consensus sequences are 9 or 10 bp in length, composed of two 5-bp half-sites. Binding as dimers, each monomer binds to one half-site in the recognition sequence, positioning the basic region into the major groove of the DNA in what has been termed the scissors grip model (Ellenberger *et al.*, 1992; Konig and Richmond, 1993; Vinson *et al.*, 1989). Importantly, in cases where the dimers are composed of two identical monomers, the DNA recognition sequence has dyad symmetry (Ellenberger, 1994).

The data presented here indicates that TOXE binds DNA in a site-specific manner, presumably via the bZIP-like basic region, despite the fact that does not contain a leucine zipper. If this is correct, then TOXE must bind DNA in a manner that is significantly different from the way true bZIP proteins function. The southwestern blot data demonstrate that TOXE binds DNA either as a monomer or as some form of homomultimer. Thus it is conceivable that TOXE monomers associate with each other to bind DNA, but only one monomer within the complex makes contact with the DNA.

Another possibility is that the lack of a leucine zipper in TOXE could be an indication that unlike the bZIP family of transcription factors which work together as dimers, TOXE may bind to DNA as a monomer. If this is true, it may explain why TOXE binds to a non-palindromic DNA sequence. At a minimum, the lack of a leucine

zipper would indicate that TOXE utilizes a different strategy for positioning the basic region onto DNA. Such a finding would not be without precedence, as Skn-1, a maternally expressed transcription factor from *Caenorhabditis elegans*, lacks a leucine zipper but has been shown to bind to a non-palindromic DNA site as a monomer via its bZIP-like basic region (Blackwell *et al.*, 1994). However, Skn-1 also contains homeodomain elements that stabilize binding and also partially determine the specificity of binding (Blackwell *et al.*, 1994; Kophengnavong *et al.*, 1999). Other than the bZIP-like basic sequence TOXE does not contain any known DNA binding motif, so it must bind DNA in a way that is also different from Skn-1.

Materials and Methods

Expression of TOXE in *E. coli*: Standard methods were used for all DNA manipulations (Ausubel *et al.*, 1989; Sambrook *et al.*, 1989). The plasmid pAJ39, containing a cDNA of the *TOXE* gene (accession no. AF038874), was identified and cloned as previously described (Ahn and Walton 1998). The 5' end of *TOXE* gene was amplified by PCR using the primers JDW623 (5'-CACGGATCCGGCACGACTTCCCCGAATAGC- 3') and JDW624 (5'-CCTTACGCTGGCTAGTTCACGAAGC-3') to create a *Bam*HI site. 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 a new plasmid called pKP9. The *TOXE* cDNA insert was removed from pKP9 with *Bam*HI and *Kpn*I and cloned between the *Bam*HI and *Kpn*I sites of the bacterial expression vector pQE30 (Qiagen, Chatsworth, Calif.) to create the TOXE expression vector pKP10. For expression, the *E. coli* strain M15 (pREP4) was transformed with pKP10 and grown overnight at 37°C in 5 ml of Luria-

Bertani medium containing ampicillin (200 μg ml^{-1}) and kanamycin (25 μg ml^{-1}). A sample of the overnight culture (250 μl) was used to inoculate 5 ml of 2XYT medium with ampicillin (200 μg ml^{-1}) and kanamycin (25 μg ml^{-1}). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM after 2.5 hours of growth to induce the expression of TOXE. After 30 minutes the cells were collected by centrifugation and the pellets were stored at -20°C.

Southwestern blot analysis: *E. coli* cell pellets were resuspended in sample buffer [0.25 M Tris-HCl (pH 6.8), 20% 2-mercaptoethanol (v/v), 8 % SDS (w/v), 30% sucrose (w/v), 0.01% bromophenol blue (w/v)] and boiled for 5 min. The denatured protein extracts were then loaded on an SDS-polyacrylamide gel (10%) and electrophoresed at 30 mA. Southwestern blot analysis was performed as described by Chen *et al.* (1993) with slight modification. The gel was equilibrated with blotting buffer [25mM Tris, 192mM glycine, 20% methanol (v/v)] for 10 min, and then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Mini Trans-Blot electrophoretic transfer cell (BioRad, Hercules, CA) following the manufacturer's instructions. The membrane was then incubated in renaturation buffer [100 mM HEPES (pH7.5), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10 mM $MgCl_2$, 5% nonfat milk] at 4°C for 18 hr. After rinsing with TNE-50 buffer [10 mM Tris (pH7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT] the blot was incubated in the same buffer containing a ^{32}P-labeled DNA probe (1.0×10^6 cpm ml^{-1}) and nonspecific competitor DNA (10 mg ml^{-1} sheared salmon sperm DNA) for 6 hr at 25°C. Then blot was then washed two times with TNE-50 at 25°C for 15 minutes followed by autoradiography.

Construction and labeling of promoter fragments for southwestern blot analysis:

Promoter fragments were amplified by PCR using the primers listed in Table 1. Reactions were carried out in 100 µl volumes with 1 ng template DNA, 10 pM of each primer (described in table 1), 3 mM $MgCl_2$, PCR reaction buffer (Life Technologies, Rockville, MD), and 1U Taq polymerase (Life Technologies) for 35 cycles (94°C, 1min; 50°C, 2 min; 72°C, 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 chromatography on a Sephadex G-50 (Sigma, St. Louis, MI) column and labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). All oligonucleotides used in this study were synthesized at the Michigan State University Macromolecular Structure Facility.

Construction and labeling of oligonucleotide probes for southwestern blot analysis:

Complementary oligonucleotides JDW-831 (5'-AACACAATCTCACGTAAGTCTG CAG-3') and JDW-832 (5'-CCTGCAGACTTACGTGAGATTGTGT-3') representing a TOXE recognition element from the *TOXA* promoter and JDW-833 (5'-AACACAGCA GGACGTAAGTCTGCAG-3') and JDW-834 (5'-CCTGCAGACTTACGTCCTGCTGT GT-3') representing a mutant version of this site were annealed by combining equimolar amounts and progressively lowering the temperature (2 min at 85°C, 15 min 65°C, 15 min at 37°C, 15 min at 25°C,

Table 1. Primers used to amplify various regions of the *TOX* gene promoters.

Name	Sequence	Gene	Direction	Fragment*
JDW-521	5'-AAA TTT CAG TTA TCT TGC AGC-3'	*TOXA*	forward	1
JDW-522	5'-TTA CTA AAG ATT CTA GCC GA-3'	*TOXA*	reverse	3
JDW-523	5'-GGA TAT TCT AAA TTA CGA TT-3'	*TOXA*	forward	2
JDW-526	5'-GAT CAG TTT GTA TGA CAG CT-3'	*TOXA*	reverse	2
JDW-703	5'-AGC TGT CAT ACA AAC TGA TC-3'	*TOXA*	forward	3
JDW-702	5'-AAT CGT AAT TTA GAA TAT CC-3'	*TOXA*	reverse	1
JDW-707	5'-GGT ACT TCT AAT GTG CCG-3'	*TOXC*	forward	1
JDW-709	5'-ACC CGA ATC ACG CTG ACC-3'	*TOXC*	forward	3
JDW-711	5'-CGT GTG GAT GGC TTC CAC-3'	*TOXC*	reverse	1 and 3
JDW-872	5'-CGG CAC ATT AGA AGT ACC-3'	*TOXC*	reverse	2
JDW-873	5'-GAG GCA GAA AGC TGA AGC-3'	*TOXC*	forward	2
JDW-614	5'-GGG GTT AAG AGC AAC ACT TAC CGT CC-3'	*TOXD*	reverse	1 and 3
JDW-874	5'-TTA GGT GAT GCT TTC TGC-3'	*TOXD*	forward	1
JDW-875	5'-GGA CTG ACA ACA TGT TGC-3'	*TOXD*	reverse	2
JDW-876	5'-GTC CGT ATT CGA TGC GTG-3'	*TOXD*	forward	2 and 3
JDW-715	5'-ATC CTA AGG CTA GGG CGT-3'	*TOXF/G*	forward	1
JDW-716	5'-CCA TGT TCG ACA TCT CGA-3'	*TOXF/G*	forward	2
JDW-717	5'-ATC CAT CTG TTG CAA CGG-3'	*TOXF/G*	reverse	1
JDW-718	5'-TAA GGC TAC GGG TGA AGG-3'	*TOXF/G*	forward	3
JDW-719	5'-AAG AAC AGC TCC TTG TCG-3'	*TOXF/G*	reverse	2and 3
JDW-720	5'-GTA AAA TGC AGG CAA GGG-3'	*TOXF/G*	reverse	4
JDW-806	5'-CTA TCT TCG ACA AGG AGC-3'	*TOXF/G*	forward	4

* Fragment numbers correspond to promoter fragments listed in Figures 2, 3, 5 and 6.

and 15 min at 4°C) using a PTC-100 programmable thermal controller (MJ Research, Waltham, MA). Double-stranded oligonucleotides were end labeled with γ-^{32}P ATP using polynucleotide kinase (New England Biolabs, Beverley, MA) as described elsewhere (Sambrook *et al.*, 1989).

Yeast strains and transformation: Standard methods were used for all manipulations of yeast cells (Guthrie and Fink, 1991). Yeast strain MG106 (MAT**a** *ade2-1 can1-100 his 3-11 15 leu 2-3 112 trp 1-1 ura 3-1*) (Myers *et al.*, 1999) was grown on either YPD (rich complete) or SD (synthetic dropout) at 28°C.

Construction of yeast reporter strains: Complementary oligonucleotides encoding either four tandem copies of the wild-type (JDW-825, 5'-tcgaATCTCACGTAATCTCAC GTAATCTCACGTAATCTCACGTA-3'; JDW-823, tcgaTACGTGAGATTACGTG AGATTACGTGAGATTACGTG-3') (lowercase letters indicate complementary ends used for cloning) or mutant versions (JDW-836, 5'-tcgaGCAGGACGTAGCAGGAC GTAGCAGGACGTAGCAGGACGTA-3'; JDW-837, 5'-tcgaTACGTCCTGCTACG TCCTGCTACGTCCTGCTACGTCCTGC-3') of the TOXE recognition sequence were ligated into the *Xho*I site of the *lacZ* reporter vector pBgl-lacZ (Li and Herskowitz, 1993). The resulting reporter constructs were integrated into the *ura*3 locus of *Saccharomyces cerevisiae* strain MG106 by transformation and selection for uracil prototrophy. *TOXE* was expressed in yeast using the pG-1expression vector (Schena *et al.*, 1991). Cells were transformed with either empty pG-1 vector or pG-1 containing a *TOXE* cDNA insert and selected for tryptophan prototrophy.

Quantitative β-Galactosidase assay: Cells from overnight cultures (2 ml) of the yeast reporter strains grown in SD media were used to inoculate 8 ml of fresh YPD. The cultures were grown at 28°C with shaking (230-250 rpm) until the cells reached an mid-log phase (OD_{600} of 1 ml = 0.3-0.5). Cells (1.5 ml) were harvested by centrifugation at 14,000 rpm for 30 sec and washed in Z-buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ (pH 7.0)]. Cells were resuspended in Z-buffer (300 µl) and disrupted using three freeze/thaw cycles. Aliquots of the disrupted cells (100 µl) were diluted with 700 µl of Z-buffer + 38 mM β-mercaptoethanol (β-Me). The substrate o-nitrophenyl β-D-galactopyranoside (ONPG) was dissolved in Z-buffer (4 mg ml^{-1}) and 160 µl was added to the disrupted cells. The reaction mixture was incubated at 30°C until a yellow color developed. The assay was terminated by the addition of 400 µl of 1 M Na_2CO_3, and the optical density (OD_{420}) of each reaction was measured with a spectrophotometer. Units of β-galactosidase were determined as using the equation [β-galactosidase units = $1000 \times OD_{420} / t \times V \times OD_{600}$; t = elapsed time (in min) of incubation; V = 0.1 ml × concentration factor; OD_{600} = OD_{600} of cultures at harvest]. A unit of β-galactosidase is defined as the amount which hydrolyzes 1µmol of ONPG to o-nitrophenol and D-galactose min^{-1} $cell^{-1}$ (Miller, 1972).

Chapter 2

SITE-DIRECTED MUTAGENESIS OF THE TOXE BZIP-LIKE BASIC REGION

Abstract

TOXE is a transcription factor that is essential for HC-toxin production by race 1 isolates of the filamentous fungus *Cochliobolus carbonum*. TOXE contains a bZIP-like basic region located near its N-terminus that is required for DNA-binding. Specific mutations within this basic region block its ability to bind DNA. TOXE also contains four ankyrin repeats located at its C-terminus. Truncated forms of TOXE lacking most or all of the ankyrin repeats were also unable to bind DNA, indicating that this portion of the protein may also be involved in binding.

Introduction

HC-toxin is a host-selective toxin produced exclusively by race 1 isolates of the filamentous fungus *Cochliobolus carbonum*. Production of HC-toxin is under the control of a single genetic locus, *TOX2*, and a single gene within this locus, *TOXE*, has been shown to be essential for the expression of other genes within this locus (Ahn and Walton, 1998). Biochemical evidence presented within the previous chapter of this dissertation indicates that TOXE is a transcription factor that recognizes and binds to conserved DNA sequences found within the promoters of genes located within the *TOX2* locus. With the demonstration that TOXE posses two distinct biochemical properties, the ability to bind DNA both *in vitro* and *in vivo*, and the ability to activate transcription in yeast, it is now possible to analyze specific regions of TOXE to determine how this protein may function.

TOXE does not appear to belong to any family of transcription factor that has been previously described. However, TOXE is predicted to contain a basic region, similar to those characteristic of the bZIP family of transcription factors (Ellenberger, 1994), near its N-terminus. In the bZIP family of proteins, the basic region plays an important role in DNA binding, as it is the portion of the protein that defines sequence specificity (Ellenberger, 1994). Based on this, it is hypothesized that the basic region of TOXE plays a similar role.

To test whether the TOXE basic region plays a role in DNA binding two approaches were taken. First, using site directed mutagenesis, conserved basic residues within this region were changed to alanines to assess their importance in DNA binding. The mutated versions of TOXE were analyzed by southwestern blot analysis, and by

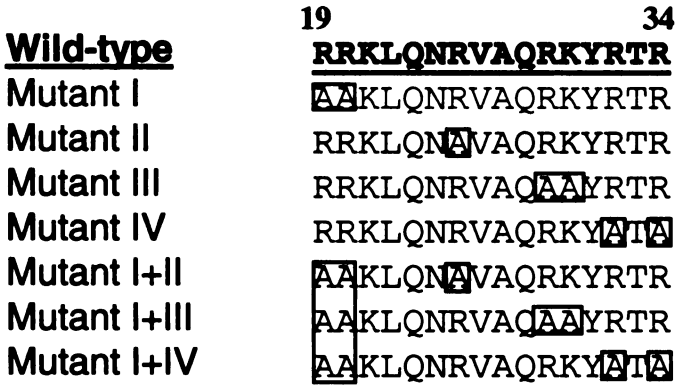
expressing them in yeast cells containing a reporter gene that is responsive to the wild-type TOXE. Second, two C-terminally truncated versions of TOXE were created to determine if a minimal DNA-binding region could be defined. The results presented here indicated that the conserved basic amino acids within the bZIP-like basic region of TOXE are important for DNA-binding. The truncation experiment failed to define a minimal region essential for DNA-binding, but raised the possibility that the four ankyrin repeats located at the C-terminal end of TOXE may also play a role in DNA-binding.

Results

Using PCR and a set of degenerate primers, the coding region of the TOXE cDNA was changed such that the most highly-conserved basic residues between positions 19 and 34 were systematically replaced with alanines. The seven mutant versions of the TOXE basic region are described in Figure 9A. With the exception of mutant III, which contains a single alanine substitution at residue 25 (R25→A), all of the mutants were designed to include at least two amino acid substitutions to increase the likelihood of detecting a phenotype. The mutant alleles of TOXE were assayed for their ability to bind DNA *in vitro* using a southwestern blot assay or *in vivo* utilizing a β-galactosidase reporter gene responsive to TOXE in yeast..

All alleles of *TOXE*, including the wild-type, were expressed constitutively in yeast strain YKP50.1 under the control of the glyceralde-3-phosphate dehydrogenase (GPD) promoter. Yeast strain YKP50.1 contains a β-galactosidase reporter gene with four tandem repeats of the tox-box within its promoter, and can be used to measure TOXE DNA binding (Figure 8A). β-galactosidase activity was determined for each of

A



B

TOXE	amino acid substitutions	β-galactosidase units
wild-type	—	72
mutant I	R19A, R20A	2
mutant II	R25A	3
mutant III	R29A, K30A	2
mutant IV	R32A, R34A	4
mutant I+ II	R19A, R20A, R25A	3
mutant I+ III	R19A, R20A, R29A, K30A	3
mutant I+ IV	R19A, R20A, R32A, R34A	2
(no expression)	—	2

Figure 9. Site-directed mutagenesis of the TOXE bZIP-like basic region. **(A)** Diagram illustrating the amino acid substitutions used to create each mutant version of TOXE. **(B)** Results of the β-galactosidase assay. Wild-type TOXE and all mutant forms were expressed in strain YKP50.1-E, which conatain a *lacZ* reporter gene that is activated by TOXE. Activity is reported in β-galactosidase units (described in methods section).

the various yeast strains containing the different alleles of *TOXE* (Figure 9B). Only the yeast strain containing the wild-type allele of *TOXE* showed significant levels of β-galactosidase activity. Although these results could be indicative that the mutant forms of TOXE were unable to bind DNA, it was also possible that the mutant forms of the protein were not expressed or not localized to the nucleus.

As a complement to the *in vivo* analysis, the ability of the mutant versions of TOXE to bind DNA was analyzed *in vitro* using southwestern blot analysis. For this experiment the mutant versions of TOXE were expressed in *E. coli*. Based on coomassie blue staining, all seven alleles were expressed at levels comparable to the wild-type protein (Figure 10A). After transferring the expressed proteins to nitrocellulose and allowing them to renature in the absence of SDS, southwestern blot analysis was performed (Figure 10B). The DNA probe used was a fragment of the *TOXA/HTS1* promoter that contained a single tox-box binding site. Whereas the wild-type TOXE bound strongly to the probe, all of the mutant versions of TOXE showed either reduced binding or no binding at all, indicating mutations within this region had an effect on DNA binding.

In many transcription factors the DNA binding activity is restricted to a relatively small portion of the protein. Based on the mutational analysis of the TOXE basic region, the N-terminal region of TOXE plays a role in DNA binding. To determine if the ankyrin repeats, located at the C-terminal end of TOXE were involved in DNA binding, two truncated forms of TOXE were constructed and expressed in *E. coli*. The truncated forms of TOXE were then assayed for DNA binding by southwestern blot analysis.

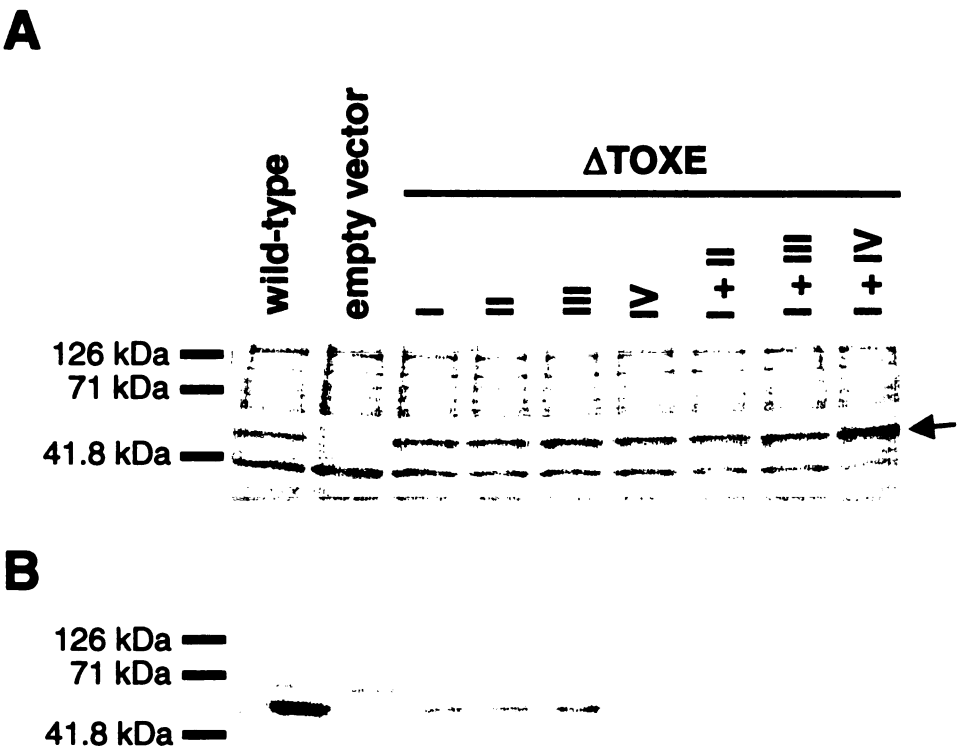


Figure 10. Southwestern blot analysis of TOXE bZIP-like basic mutants. **(A)** SDS-PAGE analysis of total protein extracts from *E. coli* cells expressing wild-type TOXE and the mutant versions. Arrow indicates the position of the recombinant proteins. **(B)** Southwestern blot analysis of the expressed proteins shown above. The contents of the gel were transferred to nitrocellulose and probed with a ^{32}P-labeled DNA fragment containing
a single tox-box.

The truncated forms of TOXE were expressed at levels comparable to the full length version of the protein in *E. coli* (Figure 11). When assayed for the ability to bind DNA, neither of the truncated forms of TOXE appeared to have retained this activity. While this may be an indication that the ankyrin repeats are required for DNA binding, it is also possible that the truncated forms of the protein are unable to renature like the full length form.

Discussion

TOXE has been shown to be required for the expression of several genes within the *TOX2* locus (Ahn and Walton, 1998; Cheng *et al.*, 1999; Cheng and Walton, 2000). Interestingly, TOXE is not predicted to fall within any established class of transcription factor. So in light of the finding that TOXE is a both a DNA-binding protein and has the capacity to function as an activator of transcription, it seemed logical to ask how the protein is capable of serving in this role.

To address the question of how TOXE specifically recognizes and binds DNA, this study focused on the bZIP-like basic region consisting of residues 19-34. This region was of particular interest because it serves to determine the DNA sequence specificity and contributes to the stabilization of binding in the bZIP class of transcription factors (Konig and Richmond, 1993).

In agreement with what has been seen for the bZIP proteins, the basic region of TOXE appears to play a key role in DNA recognition and binding. All of the mutants created by replacing the conserved basic residues in this region with alanine residues were impaired in their ability to bind DNA. The same result was found when these

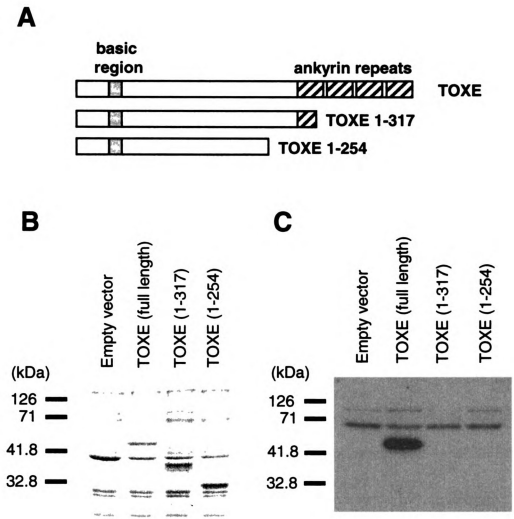


Figure 11. DNA binding assay using TOXE with the ankyrin repeats removed. **(A)** Diagram illustrating the relative positions of the bZIP-like basic region and the four ankyrin repeats. Truncated versions of TOXE are shown. **(B)** Expression of TOXE and the two truncated forms in *E. coli.* **(C)** Southwestern blot analysis using the truncated forms of TOXE. The contents of the gel were transferred to nitrocellulose and probed with a ^{32}P-labeled DNA fragment containing a single tox-box.

mutated forms of TOXE were tested *in vitro* and *in vivo*, arguing strongly that this region plays a role in the observed DNA binding activity.

In the bZIP family of transcription factors, the leucine zippers play two important roles. First, this motif allows proteins to dimerize either as homo- or heterodimers, which is required for binding to DNA and for sequence specificity. Second, by forming dimers the two basic regions adjacent to the zipper are positioned so they can easily associate with the major groove on the DNA. Since TOXE does not have a leucine zipper, it is unclear how it manages to position the basic region for binding.

Perhaps the C-terminally located ankyrin repeats play a role in this function. Although an extensive analysis of ankyrin repeats was not performed here, the data that was collected can be interpreted to signify that they may be essential for TOXE to bind DNA. It is conceivable that they may serve to stabilize binding, or perhaps to present the basic region in a manner such that it is available to bind DNA. It is also possible that the ankyrin repeats are required for TOXE monomers to form stable associations with other monomers. However, the sequence of the tox-box gives no indication that TOXE binds as anything but a monomer.

Materials and Methods

Site-directed mutagenesis: Substitutions were introduced into the *TOXE* open reading frame at specific positions by PCR using degenerate oligonucleoties (JDW-896 5'-ATT CTGAAGCTTTGCTGCCTCATTTATGTCGGT-3', JDW-893 5'-CGACGAAAG CTTCAGAATGCTGTAGCTCAAAGAAA-3', JDW-894 5'-CGACGAAAGCTTCAG AATCGTGTAGCTCAAGCAGCATACCGAACAAGGCA-3', JDW-895 5'-CGACGA

AAGCTTCAGAATCGTGTAGCTCAAAGAAAATACGCAACAGCGCAGAAAACA CGTATG-3') to create mutants I, II, III, and IV, respectively. PCR products were cloned into pKP9 (see Materials and Methods, Chapter 1) using restriction endonuclease sites present within the *TOXE* sequence. The *Hin*dIII site present between mutations I and II was used to create the double mutants (I+II, I+III, I+IV).

TOXE deletion constructs: Plasmids pKP36 and pKP37 were constructed for expressing C-terminal deletions of TOXE in *E. coli*. For pKP36, a *Bam*HI–*Eco*RV fragment containing a portion of the *TOXE* coding region was cloned into pQE30 (Qiagen, Chatsworth, Calif.). pKP37 was constructed by cloning a *Bam*HI–*Eco*RI portion of *TOXE* into pQE30.

Expression of the mutated forms of TOXE in *E. coli*:

The *TOXE* site-directed mutants and deletion constructs were cloned into the bacterial expression vector pQE30. For expression, the *E. coli* strain M15 (pREP4) was transformed with the *TOXE* expression vectors and grown overnight at 37°C in 5 ml of Luria-Bertani medium containing ampicillin (200 $\mu g\ ml^{-1}$) and kanamycin (25 $\mu g\ ml^{-1}$). A sample of the overnight culture (250 µl) was used to inoculate 5 ml of 2×YT medium with ampicillin (200 $\mu g\ ml^{-1}$) and kanamycin (25 $\mu g\ ml^{-1}$). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM after 2.5 hours of growth to induce the expression of the TOXE mutants. After 30 minutes the cells were collected by centrifugation and the pellets were stored at -20°C.

Expression of the deletion constructs and mutated forms of TOXE in yeast: Standard methods were used for all manipulations of yeast cells (Guthrie and Fink, 1991). Yeast strain YKP50.1 (see Chapter 1) was transformed with either empty pG-1 (Schena *et al.*, 1991) or pG-1 containing one of the mutated versions of *TOXE* and selected for tryptophan prototrophy.

Quantitative β-Galactosidase assay: Cells from overnight cultures (2 ml) of the yeast reporter strain YKP50.1 containing the wild-type and mutant versions of TOXE were each grown in SD media and were used to inoculate 8 ml of fresh YPD. The cultures were grown at 28°C with shaking (230-250 rpm) until the cells reached an mid-log phase (OD_{600} of 1 ml = 0.5-0.3). Cells (1.5 ml) were harvested by centrifugation at 14,000 rpm for 30 sec and washed in Z-buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ (pH 7.0)]. Cells were resuspended in Z-buffer (300 µl) and disrupted using three freeze/thaw cycles. Aliquots of the disrupted cells (100 µl) were diluted with 700 µl of Z-buffer + 38 mM β-mercaptoethanol (β-Me). The substrate o-nitrophenyl β-D-galactopyranoside (ONPG) was dissolved in Z-buffer (4 mg ml^{-1}) and 160 µl was added to the disrupted cells. The reaction mixture was incubated at 30°C until a yellow color developed. The assay was terminated by the addition of 400 µl of 1 M Na_2CO_3, the optical density (OD_{420}) of each reaction was measured with a spectrophotometer. Units of β-galactosidase were determined as using the equation [β-galactosidase units = $1000 \times OD_{420} / t \times V \times OD_{600}$; t = elapsed time (in min) of incubation; V = 0.1 ml × concentration factor; OD_{600} = OD_{600} of cultures at harvest]. A

unit of β-galactosidase is defined as the amount which hydrolyzes 1μmol of ONPG to o-nitrophenol and D-galactose min^{-1} $cell^{-1}$ (Miller, 1972).

Southwestern blot analysis: *E. coli* cell pellets were resuspended in a sample buffer [0.25 M Tris-HCl (pH 6.8), 20% 2-mercaptoethanol (v/v), 8 %SDS (w/v), 30% sucrose (w/v), 0.01% bromophenol blue(w/v)] and boiled for 5 min. The denatured protein extracts were then loaded on a SDS-polyacrylamide gel (10%) and electrophoresed at 30 mA. Southwestern blot analysis was performed as described by Chen et al. (1993) with slight modification. The gel was equilibrated with blotting buffer [25mM Tris, 192mM glycine, 20% methanol (v/v) for 10 min, and then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Mini Trans-Blot electrophoretic transfer cell (Biorad, Hercules, CA) following the manufacture's instructions. The membrane was then incubated in renaturation buffer [100 mM HEPES (pH7.5), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10 mM $MgCl_2$, 5% nonfat milk] at 4°C for 18 hr. After rinsing with TNE-50 buffer [10 mM Tris (pH7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT] the blot was incubated in the same buffer containing a ^{32}P-labeled DNA probe (1.0×10^6 cpm ml^{-1}) and nonspecific competitor DNA (10 mg/ml sheared salmon sperm DNA) for 6 hr at 25°C. Then blot was then washed two times with TNE-50 at 25°C for 15 minutes followed by autoradiography.

Construction and labeling of promoter fragments for southwestern blot analysis: For the probe, a fragment from the *TOXA* promoter containing a single tox-box was amplified using PCR with the primers JDW-525 (5'-GCGATTGTCATAGTCTCAAT-3') and

JDW-522 (5'-TTACTAAAGATTCTAGCCGA-3'). Labeling reactions were carried out in 100 µl volumes with 1 ng template DNA, 10 pM of each primer (described in table 1), 3 mM $MgCl_2$, PCR reaction buffer (Life Technologies, Rockville, MD), and 1U Taq polymerase (Life Technologies) for 35 cycles (94°C, 1min; 50°C, 2 min; 72°C, 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 chromatography on a Sephadex G-50 (Sigma, St. Louis, MI) column and labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). All oligonucleotides used in this study were synthesized at the Michigan State University Macromolecular Structure Facility.

Chapter 3

IDENTIFICATION AND MUTATIONAL ANALYSIS OF A TRANSCRIPTIONAL ACTIVATION REGION OF TOXE

Abstract

TOXE is a site-specific DNA-binding protein that activates the transcription of genes of the *TOX2* locus of *Cochliobolus carbonum*. The transcriptional activation region of TOXE, as defined by truncation analysis, is composed of 63 amino acids represented by positions 254-316 of the 441-amino acid protein. Site-directed mutagenesis of bulky hydrophobic residues within this region greatly affected the ability of TOXE to activate transcription in yeast. All but one of eight mutants made were unable to activate transcription, indicating that these residues contribute to the activation property of TOXE. In contrast, one mutant (I298→A, L300→A) showed an increase in its ability to activate transcription compared to the wild type. These results help establish the minimal activation region required by TOXE, which had not previously been characterized. Also, these results are consistent with the emerging hypothesis that bulky hydrophobic and aromatic amino acids contribute to the activational properties of transcriptional activators.

Introduction

Initiation of messenger RNA transcription is a primary control point in the regulation of gene expression. In eukaryotes, transcription of protein-coding genes is governed by the action of RNA polymerase II (Pol II), a multi-subunit enzyme that is assembled at the gene promoter. Assembly of Pol II into the pre-initiation complex, which includes several general transcription factors (GTFs) in addition to Pol II itself, can be influenced in a positive manner by the presence of activator proteins that bind to specific DNA elements within the promoter.

Activator proteins are thought to augment the transcription of specific genes through interactions with other proteins that they recruit to the promoter (Ptashne and Gann, 1997). Such interactions can involve chromatin remodeling enzymes that alter the chromatin structure when present, or basal transcription factors that participate in the formation or stabilization of the pre-initiation complex, or assist in the process of transcription itself (Struhl, 1999). Thus, an activator is operationally defined and does not indicate the mechanism of action. However, regardless of how activators work, interactions between transcriptional activators and particular target proteins are likely to require specific interactions that are dependent on the structural features of the activator protein itself.

Transcriptional activators are typically composed of separable domains that allow them to bind to DNA and domains that enable them to interact with other proteins (Tjian and Maniatis, 1994). The domains that mediate protein-protein interactions are referred to as activation domains based on their ability to activate transcription. Several different classes of activation domains have been described, based largely on their amino acid

composition, and include those rich in acidic residues, in glutamine, in proline, and in serine and threonine (Tjian and Maniatis, 1994; Triezenberg, 1995). In addition to these common classes, other activator proteins have been identified that are rich in isoleucine (Attardi and Tjian, 1993) or in basic residues (Estruch *et al.*, 1994).

The classification of activators based on amino acid composition is artificial, and does not reveal much about how these proteins function (Triezenberg, 1995). Furthermore, mutational analyses of several activator proteins have revealed that the most abundant amino acids are often not the most critical for activation activity (Sullivan *et al.*, 1998). Instead, the results of these studies have revealed that specific bulky hydrophobic or aromatic amino acids within the activation domain are often more important than the prevalence of certain types of amino acids (Sullivan *et al.*, 1998).

TOXE, a protein of 441 amino acids, is a transcriptional activator of genes required for the production of HC-toxin by the filamentous fungus *Cochliobolus carbonum*. TOXE binds specifically to an upstream DNA sequence called the tox-box and activates transcription of genes within the *TOX2* locus. The ability of TOXE to function as a transcription factor in yeast indicates that TOXE likely contains a region that serves as a transcriptional activation domain.

Sequence analysis of TOXE did not reveal the presence of an activation domain that could be easily classified into one of the known classes of transcriptional activators. Therefore, to determine how TOXE functions to activate transcription, a series of amino- and carboxy-terminal deletions were constructed and assayed for their ability to activate transcription in yeast. Once a minimal transcriptional activation region was identified,

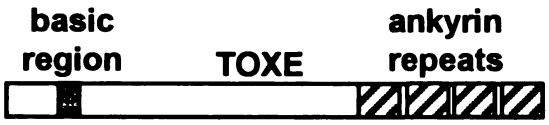
site-directed mutagenesis was used to determine the importance of specific residues within this region.

Results

Plasmids expressing various portions of TOXE fused to the Gal4 DNA-binding region were constructed and transformed into the yeast reporter strain Y190. Figure 12 summarizes the regions of TOXE used in this study. Strain Y190 contains a *GAL1-lacZ* reporter gene in the chromosome, and β-galactosidase activity was used as a measure of the transcriptional activation function of each of the TOXE fragments.

Figure 12 shows the results of a qualitative colony-lift assay performed with each of the fusion proteins. Since the level of protein synthesized in each strain was not determined, it is not possible in every case to tell if a lack of β-galactosidase activity reveals whether the fusion proteins are functionally inactive, or simply unstable. Therefore, only positive results can be taken as meaningful. Based on this analysis, a small region of 63 amino acids, representing residues 254-316 of TOXE, were found to be required for activation of the reporter gene.

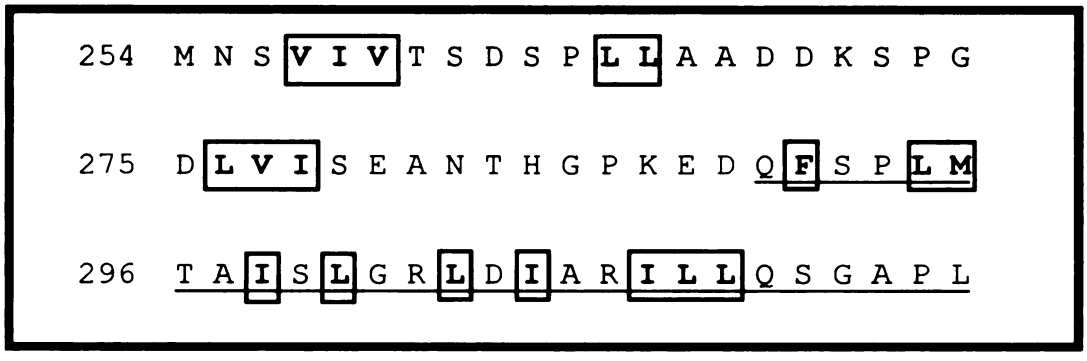
To extend the analysis of this region, site-directed mutagenesis was performed to test the relative importance of bulky hydrophobic or aromatic amino acids within this region. Figure 13 summarizes the changes made within the putative TOXE activation region, and the results of a quantitative β-galactosidase assay. With the exception of TOXE M6 (I298→A, L300→A), all mutations resulted in a loss of activity. Interestingly, the activity of TOXE M6 seemed to be enhanced, showing almost double the activity of the wild-type.



Residues of TOXE	β-galactosidase activity
1-441	+
1-254	–
167-441	+
1-155	–
1-317	+
1-336	+
1-349	+
1-432	–
167-317	+
167-336	+
167-432	–
167-254	–
167-289	–
254-317	+

Figure 12. Deletion analysis to identify a transcriptional activation domain in TOXE. Various N-terminal and C-terminal deletions of TOXE were constructed and expressed as fusion proteins with $Gal4_{DBD}$. The portion of TOXE represented by each construct is illustrated. Additionally, the amino acid positions marking the boundaries of each construct are shown. The results of a qualitative β-galactosidase assay are indicated, where (+) indicates β-galactosidase activity, and (–) indicates no activity.

A



B

TOXE	amino acid substitutions	β-galactosidase units
wild-type	—	70
mutant 1	V257A, I258A,V259	6
mutant 2	L265A, L266A	7
mutant 3	L276A, V277A, I278	14
mutant 4	F291A	10
mutant 5	L294A, M295A	2
mutant 6	I298A, L300A	125
mutant 7	L303A, I305A	4
mutant 8	I308A, L309A, L310A	8
(no expression)	—	1

Figure 13. Site-directed mutagenesis of the TOXE activation region. **(A)** Diagram illustrating the 63 residue activation region identified through deletion analysis. Amino acid residues that were changed to alanines through site-directed mutagenesis are boxed. Underlined residues constitute part of the first ankyrin repeat. **(B)** Results of the β-galactosidase assay. Changes made to the TOXE sequence for each mutant are indicated. Wild-type TOXE and all mutant forms were expressed as a Gal4DBD:TOXE in yeast strain Y190. β-galactosidase activity is reported in β-galactosidase units (described in methods section).

Discussion

The regulated expression of eukaryotic genes requires transcription factors that have both the ability to recognize specific DNA sequences within the promoters of the genes they regulate as well as the ability to associate with other proteins through specific protein-protein interactions (Tjian and Maniatis, 1994). These two disparate functions are typically mediated by separable domains, a DNA-binding domain and one or more transcriptional activation domains that function through interactions with specific target proteins. While much is known about both the structure and function of DNA-binding domains, comparatively little is known about activation domains. Often activation domains can be predicted from the sequence of a protein, as several classes of activation regions are now well established (Triezneberg, 1995). However, activation domains are functionally defined, so any meaningful prediction must be supported by biochemical data.

The fact that TOXE can activate the transcription of an otherwise silent reporter gene in yeast suggested that it likely contained an activation domain. However, no obvious domain could be inferred from the primary structure of TOXE. The most prominent feature of TOXE, based on sequence analysis, are the four ankyrin repeats located at its C-terminal end. Since the role of ankyrin repeats in protein-protein interactions is now a well-established theme in biochemistry (Sedgwick and Smerdon, 1999), it was initially hypothesized that the ankyrin repeats of TOXE may function as an activation domain.

While the results presented here do not totally discredit this hypothesis, they do suggest at least three of the ankyrin repeats are dispensable for TOXE to activate

transcription. Furthermore, both the results of the truncation analysis and the site-directed mutagenesis indicate that a portion of TOXE located directly N-terminal to the first ankyrin repeat is required for activation.

The activation region identified in this study consists of at least 63 amino acids, as this was the minimal amount of TOXE that could activate transcription in the assay employed. This region is not particularly rich in any one type of amino acid, and so does not fall into any established class of activation domain. However, more than a third of the residues within this region are hydrophobic, and most of the site-directed mutations which changed groups of these residues to alanines lost the ability to serve as activators. These findings are particularly interesting given the emerging theme that hydrophobic residues may play a key role in stabilizing interactions between activators and their targets (Sullivan *et al.*, 1995; Triezenberg, 1995).

The current biophysical data suggest that activation domains are relatively unstructured in isolation but assume more highly ordered structures that are induced in the presence of their target proteins (Sullivan *et al.*, 1995). At least part of the activation domain of TOXE identified here involves a portion of the first ankyrin repeat. Since ankyrin repeats are thought to be relatively stable structures, it is conceivable that the activation domain of TOXE may be structured even in the absence of an interacting partner. It is also possible that a highly ordered structure of the ankyrin motif may be crucial for essential hydrophobic residues to be maintained at the surface of the protein.

Perhaps the most surprising, and most intriguing result was the gain-of-activity mutant identified through site directed mutagenesis. Based on structural predictions, the alanine substitutions at positions 298 and 300 (I298→A, L300→A), fall within the first

helical segment of first ankyrin repeat. How this might lead to an increase in the ability of TOXE to activate transcription is not known.

Admittedly, the analysis of the TOXE activation domain performed here was not exhaustive, and the selection of the targets for the site-directed mutagenesis was biased by the findings of other groups (Sullivan *et al.*, 1995). It would be interesting to apply a random mutagenesis approach to determine if other residues within this region also contribute in a positive or negative manner to the function of this domain. It would also be interesting to remove the first ankyrin repeat entirely, in order to see if the second ankyrin repeat could substitute for the first. Since ankyrin repeats have not yet been implicated as transcriptional activators, such a finding would be novel.

Materials and Methods

Construction of $Gal4_{DBD}$:TOXE expression vectors: Plasmid pKP39 was used for all manipulations. This plasmid is identical to pAJ39 (Ahn and Walton, 1998) except that it has an *Nco*I site at the beginning of the coding region of *TOXE*, which was introduced by PCR. All C-terminal deletions were made by utilizing restriction sites present in the *TOXE* sequence. N-terminal deletions were constructed using PCR with primers complementary to *TOXE*. All constructs were cloned into pAS2-1 (Clontech Laboratories, Palo Alto, CA) to produce a chimeric *GAL4:TOXE* open reading frame.

Site-directed mutagenesis: Site-directed mutagenesis of *TOXE* was performed in accordance with a PCR "megaprimer" mutagenesis protocol (Sarkar and Sommer, 1990). Changes were introduced through primers complementary to *TOXE* except at the

position(s) of the desired change(s). The PCR products were then used as primers for a second round of PCR. Restriction sites present outside of the bp changes on the final product were used to replace portions of the TOXE coding region in pKP39. Mutated version *TOXE* were then subcloned into pAS2-1 for expression in yeast.

Expression of the deletion constructs and mutated forms of TOXE in yeast: Standard methods were used for all manipulations of yeast cells (Guthrie and Fink, 1991). Yeast strain Y190 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*) (Clontech Laboratories, Palo Alto, CA) was transformed with either empty pAS2-1 or pAS2-1 containing a portion of the *TOXE* coding region to produce Gal4$_{DBD}$:TOXE fusion proteins. All transformants were selected for tryptophan prototrophy.

Qualitative β-Galactosidase assay: Yeast transformants were grown on SD plates for 2-3 days at 30°C and overlaid with a nitrocellulose membrane filter (Schleicher and Schuell, Keene, NH). 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 β-D-galactoside (X-Gal) dissolved in Z-buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 38 mM β-mercaptoethanol (pH 7.0)]. The filters were then incubated at 30°C until colonies turned blue.

Quantitative β-Galactosidase assay: Cells from overnight cultures (2 ml) of the yeast reporter strain Y190 containing the wild-type and mutant versions of TOXE were each

grown in SD media and were used to inoculate 8 ml of fresh YPD. The cultures were grown at 28°C with shaking (230-250 rpm) until the cells reached an mid-log phase (OD_{600} of 1 ml = 0.5-0.3). Cells (1.5 ml) were harvested by centrifugation at 14,000 rpm for 30 sec and washed in Z-buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ (pH 7.0)]. Cells were resuspended in Z-buffer (300 µl) and disrupted using three freeze/thaw cycles. Aliquots of the disrupted cells (100 µl) were diluted with 700 µl of Z-buffer + 38 mM β-mercaptoethanol (β-Me). The substrate o-nitrophenyl β-D-galactopyranoside (ONPG) was dissolved in Z-buffer (4 mg ml^{-1}) and 160 µl was added to the disrupted cells. The reaction mixture was incubated at 30°C until a yellow color developed. The assay was terminated by the addition of 400 µl of 1 M Na_2CO_3, the optical density (OD_{420}) of each reaction was measured with a spectrophotometer. Units of β-galactosidase were determined as using the equation [β-galactosidase units = $1000 \times OD_{420} / t \times V \times OD_{600}$; t = elapsed time (in min) of incubation; V = 0.1 ml × concentration factor; OD_{600} = OD_{600} of cultures at harvest]. A unit of β-galactosidase is defined as the amount which hydrolyzes 1µmol of ONPG to o-nitrophenol and D-galactose min^{-1} $cell^{-1}$ (Miller, 1972).

Chapter 4

REGULATION OF *HTS1* BY TOXE

Introduction

Highly virulent race 1 isolates of the maize pathogen *Cochliobolus carbonum* produce HC-toxin. Production of the toxin appears to be governed by a single genetic locus, *TOX2* (Walton, 1996). Synthesis of the toxin is catalyzed by at least four enzymes, encoded by the genes *HTS1*, *TOXC*, *TOXF* and *TOXG*, which are found exclusively in toxin-producing isolates of the fungus (Ahn and Walton, 1997; Cheng *et al.*, 1999; Cheng and Walton, 2000; Pannacione *et al.*, 1992; Scott-Craig *et al.*, 1992). *TOXA* and *TOXE*, which are also part of the *TOX2* locus, are also thought to be required for secretion of the toxin and the transcriptional regulation of genes within the locus, respectively (Pitkin *et al.*, 1996; Ahn and Walton, 1998).

Infection of maize by *C. carbonum* typically begins with the germination of asexual fungal spores, called conidia, on the leaves of susceptible plants. Since HC-toxin is not stored in ungerminated conidia, it must therefore be produced *de novo* before or during the infection process (Dunkle *et al.*, 1991). Using plasma desorption mass spectrometry (PDMS), which is capable of detecting as little as 0.5 ng of HC-toxin, Weierigang *et al.* (1996) were able to show that HC-toxin is synthesized within 4 hrs after conidial germination.

Central to the production of HC-toxin is HC-toxin synthetase (HTS), a large 570-kDa multi-subunit enzyme (Walton and Holden, 1988), the product of the 15.7-kb *HTS1* gene (Pannacione *et al.*, 1992; Scott-Craig *et al.*, 1992). Transcripts for *HTS1* can be

detect as early as 3 hr post-germination (Jones and Dunkle, 1995). Unlike the other genes of the *TOX2* locus, *HTS1* has not been reported to be regulated by *TOXE*. On the contrary, enzyme activity for *HTS1* appeared to increase in strains of the fungus that lacked a functional copy of *TOXE* (Ahn and Walton, 1998).

The transcriptional start site of *TOXA*, which was shown to be regulated by *TOXE* (Ahn and Walton, 1998), is only 386 bp away from the transcriptional start site of *HTS1* and is transcribed in the opposite orientation from a shared promoter region (Pitkin *et al.*, 1996). Given that (1) both genes are unique to the *TOX2* locus, (2) *TOXF* and *TOXG* have a similar promoter architecture as *HTS1/TOXA* and are both regulated by *TOXE*, and (3) TOXE binds to recognition sites in the promoters of *TOXF/TOXG* and *HTS1/TOXA*, it seemed curious that they would not be regulated in a similar manner. However, such a finding would not be without precedence. *acvA* and *ipnA*, two genes of the *Apergillus nidulans* penicillin biosynthetic gene cluster, are also divergently transcribed from a shared promoter yet do not appear to be coordinately regulated (Then Bergh *et al.*,1996).

To test whether *HTS1* might be regulated in the same fashion as the other genes within the *TOX2* locus, RT-PCR was used. RNA from a wild-type race 1 strain was compared with RNA from a strain that lacks a functional copy of *TOXE* using primers specific for *HTS1*, *TOXA*, and *GPD*. Both *HTS1*and *TOXA* transcripts were only detected in the wild-type strain, whereas *GPD*, which is not expected to be regulated by *TOXE*, was detected in both strains.

Results and Discussion

Total RNA was isolated from two strains, 164R10 (wild-type) and T647, a transformed isolate of 164R10 in which the single copy of *TOXE* had been disrupted (Ahn and Walton, 1998). Total RNA was treated with RNase-free DNase prior to further manipulations. First strand synthesis was performed under standard conditions using primers designed to amplify portions of *HTS1*, *TOXA*, and *GPD*. As a control to detect DNA contamination, a second set of reactions were carried out omitting the reverse transcriptase. A fraction of the first reaction was then used as the template for PCR. The products of the PCR were then separated on a 1.7% agarose gel. The primers used for PCR were designed to produce bands of 191 bp, 106 bp, and 159 bp for *HTS1*, *TOXA*, and *GPD*, respectively.

Comparison of the reactions using either 164R10 or T647 RNA showed significant differences (Figure 14). Bands representing a portion of all three genes were produced when the RNA from 164R10 was used as the starting material. In contrast, only *GPD* was detected from the T647 RNA. This was taken as evidence that both *HTS1* and *TOXA* were down-regulated in the absence of TOXE, and that *GPD* transcript levels were unaffected.

Given that the shared promoter between *HTS1* and *TOXA* contains two binding sites for TOXE, it is perhaps logical to assume that TOXE would regulate the expression of *HTS1*. However, this is in direct contrast to the findings of Ahn and Walton (1998) who reported that the levels of HTS activity increased when *TOXE* was disrupted. This discrepancy has yet to be reconciled. Testing the transcript of *HTS1* gives a more direct measure of gene regulation than does measuring the enzyme activity.

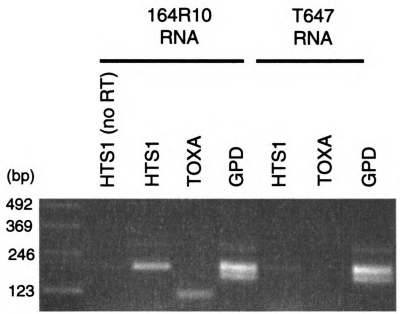


Figure 14. Expression of *HTS1*, *TOXA*, and *GPD* in the wild-type (164R10) and in the *TOXE* mutant (T647-1). Products of the RT-PCR were separated on a 1.7% agarose gel.

It is possible that the level of another enzyme that caused false-positive results increased in the absence of TOXE.

Materials and methods

Fungal strains: *C. carbonum* strains were stored and cultured as previously described (Walton, 1987). Strain 164R10 ($Tox2^+$) resulted from a cross between two standard laboratory strains, SB111 ($Tox2^+$) and SB114 ($Tox2^-$). T647 was derived from 164R10 and is identical except that *TOXE* has been disrupted (Ahn and Walton, 1998).

RNA isolation: For RNA isolation, *C. carbonum* strains were grown for six days on modified Fries' medium supplemented with 2% (w/v) sucrose as the carbon source. Total RNA was isolated from lyophilized mycelial mats by the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987).

RT-PCR: Total RNA was DNase treated with RNase-free DNase for 1 hr at 37°C. The enzyme was then inactivated by heating the reactions to 70°C for 15 min. First strand synthesis was carried out at 42°C for 1 hr using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Life Technologies, Rockville, Maryland) using primers specific for *HTS1* (JDW-1237, 5'-GGCGGGTAGCTATCAAGTATTGTG-3'), *TOXA* (JDW-1242, 5'-ATAAAGAGCCATTTGGGCG-3'), and *GPD* (JDW-1243, 5'-CTCGCTCCATGGAATGTT-3'). The product of the first strand synthesis was then used as the template for PCR, using standard conditions with an annealing temperature of 55°C. Primers used were JDW-1242 and JDW-971 (5'-GGCTGCCTTGTCGAAGCA-

3') for *HTS1*, JDW-973 (5'-CTCGATGCGACCGTTGTTG-3') and JDW-1249 (5'-GTGGTTCCGCTCATGAGC-3') for *TOXA*, and JDW-1238 (5'-GAGCACAACGACGTCGAC-3') and JDW-1239 (5'-GGAAACGGATGGTCTTGC-3') for *GPD*. PCR products were analyzed on a 1.7% agarose gel.

CONCLUSIONS

Eukaryotic cells contain a wide array of proteins that are able to interact with DNA. These include proteins that function in DNA replication, packaging, repair, restriction and modification, basal transcription, and transcriptional control. To function, these proteins must have the ability to bind DNA, often in a sequence-dependent manner. The analysis of DNA binding domains within these proteins has revealed many different structural motifs that enable them to function. These motifs include the helix-loop-helix, homeodomain, POU-homeodomain, high mobility group (HMG) box, MADS box, ATTS domain, basic region-leucine zipper (bZIP), and the cysteine-rich metallocoordination domains which include the C2H2, $C2X_{17}C2$ (GATA), and Zn(II)2Cys6 binuclear and ring fingers (Klug and Rhodes, 1987; Sturm and Herr, 1988; Berg, 1989; Murre *et al.*, 1989; Vinson *et al.*, 1989; Andrianopolos and Timberlake, 1991; Gehring, 1992; Merika and Orkin, 1993; Treisman, 1995; Todd and Andrianopoulos, 1997). Thus, DNA binding proteins are typically classified by the type of structural motif that enables them to function.

TOXE appears to be a pathway-specific transcription factor that controls the expression of the *TOX2* locus in *Cochliobolus carbonum*. It binds DNA in a sequence-specific manner, recognizing a conserved element (the tox-box) present in the promoters of the *TOX2* genes, and activates their expression. However, TOXE does not fit into any of the established classes of DNA binding proteins, as it lacks a known binding domain.

Although TOXE is structurally different from the known transcription factors, it does contain two motifs that are frequently found in these proteins. Near its N-terminus TOXE contains a region rich in basic residues that matches the consensus sequence for

the bZIP proteins (Bairoch *et al.*, 1995). This region is required for DNA binding and probably contributes to its specificity. However, unlike the bZIP proteins TOXE does not contain the canonical leucine zipper motif that defines the class. TOXE also differs from the bZIP proteins in that it contains an ankyrin repeat motif at its C-terminal end. Thus TOXE appears to be fundamentally different from the bZIP proteins.

TOXE may represent a new class of DNA binding protein. Recently, a gene from *Cladosporium fulvum*, *Bap1*, was identified that shows a striking degree of similarity to *TOXE*. *Bap1* is also predicted to encode a protein that contains a bZIP basic region with no adjacent leucine zipper and it is also predicted to have C-terminal ankyrin repeats (H.-J. Bussink and R. Oliver, personal communication). Furthermore, the basic region of both TOXE and Bap1 are most similar to the Yap family of bZIP proteins, which are only found in fungi (Fernandes *et al.*, 1997). Based on these observations a new class, called bANK proteins, has been proposed to describe them (H.-J. Bussink and R. Oliver, personal communication).

Regulation of the *TOX2* locus plays an important role in pathogenicity, as the expression of these genes leads to a highly virulent compatible interaction on certain maize inbred lines (Walton, 1996). Thus, the expression of *TOXE* plays a fundamental role during infection. However, nothing is known about what might regulate the expression of *TOXE*.

HC-toxin production appears to be constitutive when the fungus is grown in still culture. Under these conditions transcripts for *HTS1* appear to be up-regulated within the first 3 hrs after conidium germination (Jones and Dunkle, 1995). Therefore, *TOXE* is likely to be expressed during early stages of infection. When grown in aerated, shake

culture, HC-toxin cannot be detected, yet the *HTS1* mRNA still accumulates (Weiergang *et al.*, 1996) indicating that *TOXE* is also expressed.

It is possible that negative regulation of HC-toxin production is important, either during part of the infection process, or during the life cycle of *C. carbonum*. The fact that HC-toxin may not be down regulated is equally plausible. In culture, race 1 and race 2 isolates are indistinguishable, indicating that under these conditions production of the toxin does not appear to be energetically costly. However, it is interesting to note that in surveys of field populations, toxin-producing strain accounted for less than 2% of the individuals (Leonard, 1978), so perhaps in the absence of susceptible maize lines production of the toxin is a disadvantage. A third possibility relies on the assumption that *C. carbonum* acquired all or part of the *TOX2* locus through a horizontal transfer. If such an event did occur, perhaps the regulation of *TOXE* played an important role in another system.

Aside from the regulation of TOXE expression, other questions still remain open. In this study, two possible roles for the ankyrin repeats of TOXE were presented. The first ankyrin repeat appears to be involved in activation gene expression, an all or part of this region may be involved in DNA binding. It is also possible, given the known functions of other ankyrin repeats (Sedgwick and Smerdon, 1999), that TOXE interacts with other unknown proteins that may not be directly related to either of these two activities. Currently, the *TOX2* locus is somewhat loosely defined, so it is possible that there are still other genes related to HC-toxin production that await discovery. Given that all of the known genes of the *TOX2* locus are regulated by TOXE, it may be possible to

use the TOXE disrupted strain to isolate these genes. This could be accomplished using one of the many existing methodologies designed to detect differential gene expression.

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