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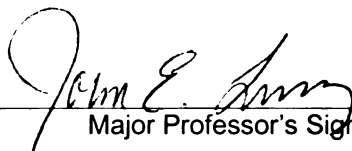
Transcriptional regulation of the *Aspergillus parasiticus*
aflatoxin biosynthetic pathway gene nor-1

presented by

Michael Joseph Miller

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Food Science and
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TRANSCRIPTIONAL REGULATION OF THE *ASPERGILLUS PARASITICUS*
AFLATOXIN BIOSYNTHETIC PATHWAY GENE *NOR-1*

By

Michael Joseph Miller

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF THE *ASPERGILLUS PARASITICUS* AFLATOXIN BIOSYNTHETIC PATHWAY GENE *NOR-1*

By

Michael Joseph Miller

Aflatoxin is a potent hepatocarcinogen produced predominantly by *Aspergillus flavus* and *A. parasiticus* that can contaminate several commodities including corn, cotton, peanuts and certain tree nuts. Aflatoxin biosynthesis is a complex process that requires several genes that all reside in an aflatoxin gene cluster. Since it would be unpractical to investigate the regulation of all aflatoxin genes, we have chosen *nor-1* to serve as a model aflatoxin gene. Nor-1 catalyzes the conversion of the first stable intermediate, norsolorinic acid to averantin. An early pathway gene, such as *nor-1*, is an ideal target for transcriptional regulation studies. In addition, there were several tools available including an *A. parasiticus nor-1* mutant strain, Nor-1 recombinant protein, polyclonal anti-Nor-1 antibody and a *nor-1* reporter strain.

The specific objectives of these studies were to: 1) demonstrate that transcriptional activation of *nor-1* (and presumably the other aflatoxin structural genes) is at least partly responsible for the increase in aflatoxin production under inducing conditions; 2) develop a *nor-1* reporter system for use in identifying *cis*-acting sites in the *nor-1* promoter; 3) determine the role of an aflatoxin pathway regulator, AflR, in the regulation of *nor-1*; and 4) identify additional possible *cis*-acting sites in the *nor-1* promoter. To measure *nor-1* transcriptional activation, plasmids that contained the *nor-1* promoter fused to the β -glucuronidase gene were transformed into *A. parasiticus*.

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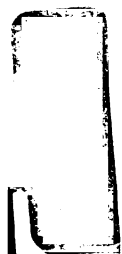
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Preliminary experiments utilized *A. parasiticus* D8D3, a strain that carries a 3.0 kb *nor-1* promoter fragment fused to GUS that integrated at the *nor-1* terminator. The transcriptional activation of *nor-1* mirrored the accumulation of aflatoxin, *nor-1* transcript and Nor-1 protein. In addition, under culture conditions that generated the most aflatoxin, the highest GUS activities were recorded. A new *nor-1::GUS* reporter plasmid was constructed that enabled easy *nor-1* promoter insertions. However, the site of integration of the *nor-1::GUS* plasmid was demonstrated to be an important consideration. Integration outside of the aflatoxin gene cluster resulted in significantly reduced *nor-1* transcription. Of the three putative AflR binding sites located in the *nor-1* promoter area (AflR1, AflR2 and AflR3), only AflR1 is clearly necessary for *nor-1* transcriptional activation. AflR2 may also be involved in *nor-1* transcriptional activation but the presence of the ORF3 gene (unknown function), which is located between *nor-1* and AflR2, prevents a clear conclusion. AflR3 is not necessary for *nor-1* transcriptional activation because deletion of AflR3 in a *nor-1::GUS* reporter strain resulted in no change in *nor-1* transcriptional activation. Substitution of a putative TATA box in the *nor-1* promoter in the context of a larger promoter demonstrated the requirement for the TATA box in *nor-1* transcriptional activation. Evidence was also presented for two additional *cis*-acting sites in the *nor-1* promoter, norL and CRE. While the studies described in this dissertation have accomplished the objectives, they have also generated several new questions. Future scientists interested in aflatoxin gene regulation have several possible research avenues including: 1) identify CRE and norL binding proteins, 2) investigate the mechanisms of aflatoxin gene-cluster-dependent regulation, and 3) investigate the function of ORF3.

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To my parents, for all their unconditional love and support

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LIST OF ABBREVIATIONS

GMS	glucose + minimal salts medium; aflatoxin inducive
PMS	peptone + minimal salts medium; aflatoxin non-inducive
YES	yeast extract + sucrose medium; aflatoxin inducive
<i>nor-1</i>	aflatoxin biosynthetic gene
<i>ver-1</i>	aflatoxin biosynthetic gene
<i>pksA</i>	aflatoxin biosynthetic gene; polyketide synthase
ORF3	open reading frame 3; located between <i>pksA</i> and <i>nor-1</i> in <i>Aspergillus parasiticus</i>
<i>aflR</i>	aflatoxin biosynthetic gene; pathway transcriptional activator
AflR1	AflR binding site in <i>nor-1</i> promoter; -65 from <i>nor-1</i> transcriptional start site
AflR2	AflR binding site in <i>nor-1</i> promoter; -1205 from <i>nor-1</i> transcriptional start site
AflR3	AflR binding site in <i>nor-1</i> promoter; -1553 from <i>nor-1</i> transcriptional start site
CRE1	potential <i>cis</i> -acting site in the <i>nor-1</i> promoter
CREbp	protein that binds CRE1
norL	potential <i>cis</i> -acting site in the <i>nor-1</i> promoter
NorLbp	protein that binds norL
EMSA	electrophoretic mobility shift assay
nor-R	PCR amplified fragment from <i>nor-1</i> promoter; contains AflR1 and CRE1

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CHAPTER 1

LITERATURE REVIEW

Background and Significance

Aspergillus and Aflatoxin

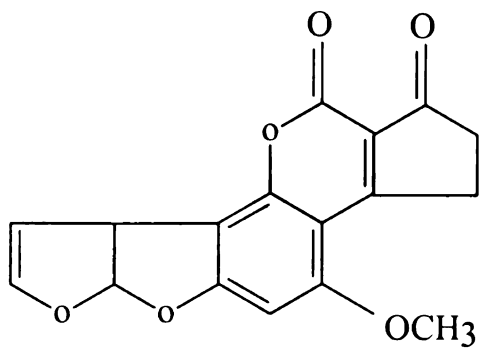
Aflatoxins are highly toxic and carcinogenic secondary metabolites of certain strains of *Aspergillus parasiticus*, *A. flavus*, *A. nomius* and *A. psuedotamarii* with only *A. parasiticus* and *A. flavus* being economically important (Council for Agricultural Science and Technology, 2003). Growth and production of aflatoxins require environmental conditions that are usually found in tropical and subtropical regions but may also be found in more temperate areas such as the United States (Dvorackova, 1990). Several different crops have been found to be contaminated with aflatoxins including corn, cotton, peanuts and certain tree nuts (Council for Agricultural Science and Technology, 2003). Infection of the plant by toxigenic *Aspergilli* can occur before harvest or during storage after harvest (Wilson and Payne, 1994). Seventeen different compounds have been isolated and designated as aflatoxins yet the term usually refers to four metabolites of this group (Figure 1.1): aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂) (McLean and Dutton, 1995). Toxigenic *A. parasiticus* typically produces all four compounds while toxigenic *A. flavus* only produces AFB₁ and AFB₂ (Dvorackova, 1990). AFB₁ is usually the most concentrated in food samples followed by AFG₁, AFB₂ and AFG₂ (McLean and Dutton, 1995). Unfortunately, AFB₁ is also the most toxic, carcinogenic, and mutagenic of the four major aflatoxins (Roebuck and Maxuitenko, 1994).

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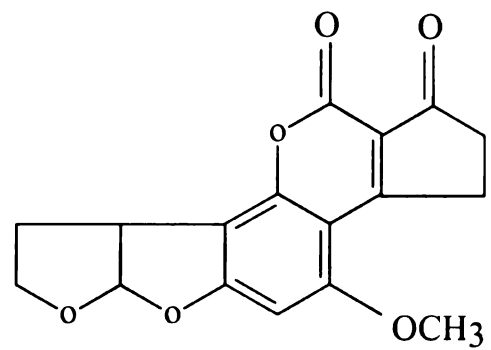
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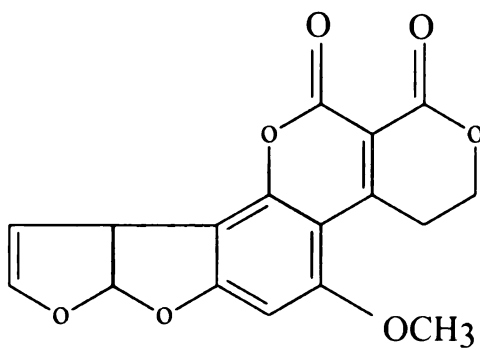




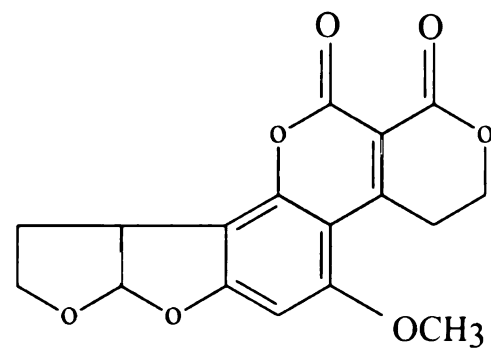
AFB₁



AFB₂



AFG₁



AFG₂

Figure 1.1. Molecular structure of the primary aflatoxins.

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Toxicology and Epidemiology

In 1960, an outbreak of acute hepatotoxic disease killed more than 100,000 turkeys (Blount, 1961). The etiologic agents were identified as *A. flavus* metabolites that were subsequently characterized and named aflatoxins (*A. flavus* toxins) (Asao et al. 1963). Since their discovery in the early 1960's, aflatoxin toxicity has been a subject of intense investigation. Aflatoxins, collectively, have been described as being a “quadruple threat” toxin – a potent toxin, mutagen, carcinogen and teratogen (McLean and Dutton, 1995). The order of acute and chronic toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (McLean and Dutton, 1995). The order of toxicity reflects the importance of the 8,9 double bond and also the greater potency associated with the cyclopentenone ring of the B series when compared to the lactone ring of the G series (McLean and Dutton, 1995). AFB_1 requires metabolic activation in order to reach its toxic and carcinogenic potential (McLean and Dutton, 1995). The 8,9 double bond in AFB_1 is epoxidated by mixed-function mono-oxygenases to the reactive form which can form adducts with cellular macromolecules including DNA, RNA, and proteins (Figure 1.2) (McLean and Dutton, 1995).

Epidemiological data have linked aflatoxins with human hepatic cancer, primarily in under-developed countries where the elimination of contaminated products is not practiced (Reviewed in Dvorackova, 1990, Hall and Wild, 1994 and Council for Agricultural Science and Technology, 2003). Initially, many researchers noticed a striking correlation between regions with high aflatoxin contamination of food products and hepatic cancer. The prevalence of viral hepatitis and other mycotoxins in these high liver cancer areas complicated the epidemiological data and prevented epidemiologists from concluding unequivocally that AFB_1 by itself is a human carcinogen (Hall and

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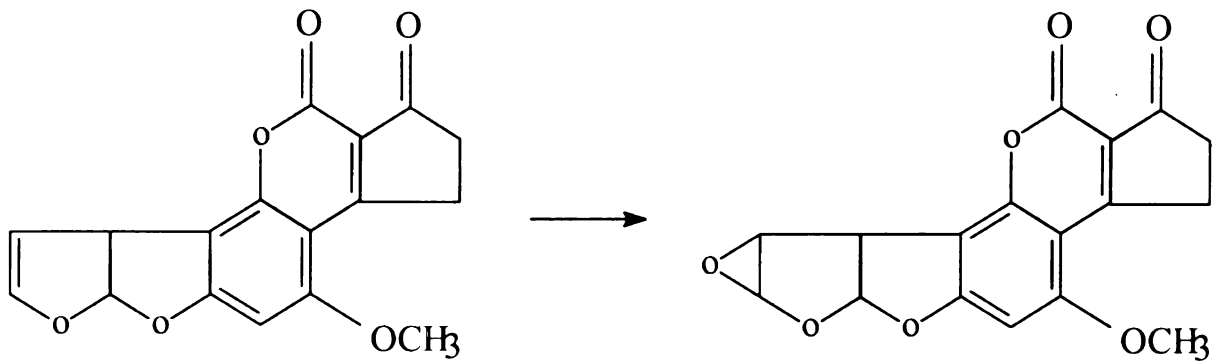


Figure 1.2. Enzymatic activation of AFB₁. The 8,9 double bond of AFB₁ is epoxidated by mixed function mono-oxygenases to the reactive epoxide. The epoxide can then form adducts with cellular macromolecules including DNA.

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Wild, 1994). With the development of biomarkers for aflatoxin exposure, such as aflatoxin adducts in urine, the etiology of liver carcinomas could be further explored. The mutational spectrum of aflatoxin is dominated by one genetic change: the GC to TA transversion (Smela et al., 2001). In fact, aflatoxin exposure has been linked to a specific transversion (GC to TA) in the tumor suppressor gene, *p53* (third position of codon 249) (Council for Agricultural Science and Technology, 2003). Use of specific biomarkers for aflatoxin exposure has demonstrated that AFB₁ and hepatitis B interact as risk factors for human liver cancer (Scholl et al., 1995). In addition, aflatoxin carcinogenicity studies clearly demonstrate the high potency of AFB₁ in several species of lab animals (reviewed in World Health Organization, 1979). Consequently, the elimination of human exposure to aflatoxin is a worthwhile goal.

Economic Costs of Aflatoxin Contamination

Due to valid health concerns, many countries have set regulatory action levels for aflatoxin in food and feed. In the United States, the action level is 20 ppb in food crops with higher action levels in feed crops (Council for Agricultural Science and Technology, 2003). Determining the precise economic cost of these action levels is not possible due to various uncertainties which include the extent and level of contamination, variability of contamination, variability of the price and quantity of the affected commodity, costs of efforts to mitigate contamination and loss in livestock value from contaminated feed (Council for Agricultural Science and Technology, 2003). Vardon et al. used a Monte Carlo computer simulation to estimate the yearly cost of aflatoxin contamination in the United States (Council for Agricultural Science and Technology, 2003). They estimated that the mean simulated potential value of crops lost because of aflatoxin contamination

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was \$47 million per year in food crops (peanut and corn), \$ 225 million per year in feed corn, and \$4 million per year in livestock cost (Council for Agricultural Science and Technology, 2003). Using a different method, Robens calculated that aflatoxin management costs the United States at least \$100 million a year (Robens, 2001). While these values are estimates, it is clear that aflatoxin contamination has a significant economic impact in the United States.

There are substantial economic costs to under-developed countries as well. At a conference in May, 2001, the United Nations Secretary-General Kofi Annan said, "... a World Bank study has calculated that the European Union regulation on aflatoxins costs Africa \$750 million each year in exports of cereals, dried fruit and nuts. And what does it achieve? It may possibly save the life of one citizen of the European Union every two years ... Surely a more reasonable balance can be found" (Annan, 2001). The low aflatoxin action levels in many countries including the United States and the European Union have reduced aflatoxin exposure yet there are large economic costs associated. Economically feasible methods to reduce or eliminate aflatoxin exposure are clearly needed.

Aflatoxin Biosynthesis - General Review

A potential means to accomplish the goal of aflatoxin elimination is to elucidate the molecular mechanisms that regulate aflatoxin biosynthesis. This information is likely to generate novel approaches and targets for inhibition of aflatoxin gene expression. The biosynthetic pathway for AFB₁ has been elucidated through the use of feeding studies, pathway mutants and inhibitors (reviewed in Bhatnagar et al., 1994). At least 16 different enzymatic steps are required for AFB₁ synthesis (Bhatnagar et al., 1991). A pair

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of aflatoxin associated fatty acid synthases, Fas-1 and Fas-2, together form the hexanoate starter molecule which is then acted on by a polyketide synthase (*pksA*) to form the first stable intermediate, the decaketide norsolorinic acid (Watanabe et al., 1996). Several enzymes responsible for the various biochemical steps have now been identified including Nor-1, Ver-1, and OmtA (Yu et al., 1995b).

Different methods have been used to clone the biosynthetic genes including reverse genetics (Yu et al., 1993), subtractive hybridization (Feng et al., 1992) and complementation (Chang et al. 1992 and Skory et al. 1992). Complementation takes advantage of the many pathway mutants that are available and was used to clone the *nor-1* (Chang et al. 1992), *ver-1* (Skory et al., 1992), *aflR* (Payne et al., 1993) and *fas-1A* (Mahanti et al., 1996) genes. Skory et al. identified one cosmid that contained both the *nor-1* and *ver-1* genes which suggested that the genes in the pathway may be clustered (Skory et al., 1992). Mapping of this cluster identified several different transcripts that have the same timing of expression as *nor-1* and *ver-1* and the genes that encode many of these transcripts have been subsequently identified and cloned (Trail et al., 1995b). The aflatoxin gene cluster from *A. parasiticus* is presented in Figure 1.3.

nor-1 - An Aflatoxin Biosynthetic Structural Gene

Using the *A. parasiticus* norsolorinic acid accumulating strain B62 (*niaD*, *nor-1*), *nor-1* was cloned by complementation (Chang et al., 1992). Subsequently, gene disruption of *nor-1* in wild type *A. parasiticus* resulted in the accumulation of norsolorinic acid supporting the proposed function of Nor-1 protein (Trail et al., 1994). The transcriptional start site and the polyadenylation site for *nor-1* were determined (Trail et al., 1994). Nucleotide sequence analysis of *nor-1* revealed that it encodes an alcohol dehydrogenase

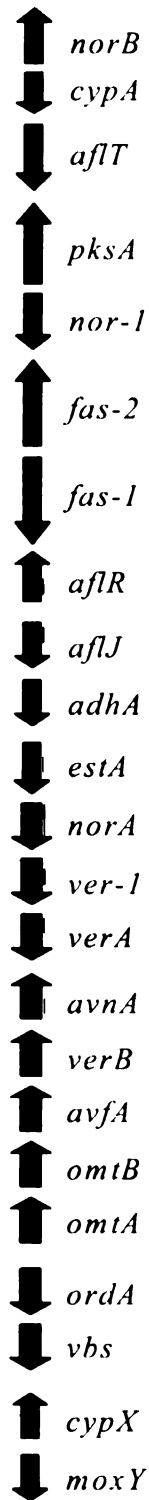


Figure 1.3. Genomic organization of the aflatoxin biosynthetic gene cluster in *Aspergillus parasiticus*. Arrowheads indicate the direction of transcription. Drawn approximately to scale.

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domain consistent with its proposed function of reducing norsolorinic acid to averantin (Trail et al., 1995a). Functional analysis with recombinant Nor-1 demonstrated that it is capable of converting norsolorinic acid to averantin *in vitro* confirming the proposed function (Zhou and Linz, 1999).

At least 20 different genes are directly involved in aflatoxin biosynthesis. An understanding of how the early pathway genes such as *nor-1* are regulated could be more effective in helping develop methods to reduce aflatoxin contamination. Since it would be impractical to study the transcriptional regulation of all aflatoxin genes, we chose to study *nor-1* specifically. The timing of expression of *nor-1* transcript and protein accumulation was consistent with the occurrence of aflatoxin (Skory et al., 1993). A reporter gene has been constructed with the *nor-1* promoter fused to the β -glucuronidase gene (*uidA*) from *Escherichia coli* (Chiou et al., 2002). Prior to the work performed in this dissertation, no specific transcription factors or their binding sites have been identified in the *nor-1* promoter.

Transcriptional Regulation of Mycotoxin Biosynthesis

Mycotoxins are secondary metabolites produced by several species of fungi. As secondary metabolites, mycotoxins are not constitutively synthesized. Rather, mycotoxin biosynthesis is a complex process that responds to developmental, environmental and nutritional cues. The primary means that fungi use to regulate mycotoxin biosynthesis appear to be transcriptional regulation of the mycotoxin biosynthetic genes. Aflatoxin and trichothecene biosynthesis are the most characterized of the mycotoxins.

Information gathered about aflatoxin and trichothecene biosynthesis has been helpful for researchers studying other mycotoxin biosynthetic pathways. In the following sections,

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the regulation of aflatoxin and trichothecene biosynthesis gene transcription is reviewed with brief mention of other mycotoxin pathways.

Regulation of Aflatoxin Gene Transcription

AflR - Discovery

A. flavus strain 650 contains a mutation in the *afl2* locus (Bennett and Papa, 1988). The parental strain for *A. flavus* strain 650 was a norsolorinic acid accumulator while the *A. flavus* 650 strain did not accumulate norsolorinic acid (Bennett and Papa, 1988). Metabolite feeding studies and enzyme activity measurements of *A. flavus* strain 650 demonstrated that the aflatoxin biosynthetic enzymes were not present in the strain and that perhaps a mutation in a regulatory gene was responsible for the phenotype (Payne et al., 1993). The gene was identified in *A. flavus* by complementation and named *aflR* (Payne et al., 1993). Subsequently, *aflR* was identified in *A. parasiticus* (Chang et al., 1993) and *A. nidulans* (Yu et al., 1996).

AflR - Function

Several pieces of biochemical evidence indicate a regulatory role for *aflR*. Metabolite feeding studies and enzymatic activity measurements with *A. flavus* 650 (*aflR* mutant) and wild type *A. flavus* demonstrated that *aflR* is necessary for the aflatoxin biosynthetic enzyme activities to be detected (Payne et al., 1993). Later, studies demonstrated that *aflR* is required for aflatoxin biosynthetic gene transcript accumulation (Yu et al., 1996). Insertion of an additional copy of *aflR* into an *A. parasiticus* O-methylsterigmatocystin producing strain resulted in the overproduction of pathway intermediates including O-methylsterigmatocystin (Chang et al., 1993). In addition,

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insertion of an additional copy of *aflR* in *A. parasiticus* resulted in the transcription of pathway genes under aflatoxin non-inducing conditions (Chang et al., 1995). Use of *aflR* linked with an inducible promoter in *A. nidulans* demonstrated that induction of *aflR* in aflatoxin non-inducing conditions can activate genes in the biosynthetic pathway (Yu et al., 1996). Based on amino acid sequence identity, AflR belongs to a common class of fungal transcription factors called zinc binuclear cluster proteins that includes Gal4 (Woloshuk et al., 1994). While the amino-terminus of AflR contains the DNA binding domain, the carboxyl-terminus of AflR has a highly acidic region that functions as a transactivation domain (Chang et al., 1999). However, the total acidity in this region is not a major determinant in AflR transactivation (Chang et al., 1999).

DNA binding by AflR has also been investigated. Using methylation interference footprinting and electrophoretic mobility shift assays, the palindromic AflR binding site was first identified as TCGNNNNCGA in the *stcU* promoter in *A. nidulans* (Fernandes et al., 1998). The consensus AflR binding site was later defined as TCGSWNNSCGR (S = C/G, W = A/T, R = A/G) based on *in vitro* binding studies with recombinant AflR (Ehrlich et al., 1999b). Recombinant AflR binds to several aflatoxin biosynthetic gene promoters *in vitro* (Fernandes et al., 1998, Ehrlich et al., 1999a, Ehrlich et al., 1999b and Miller et al., 2003a). AflR *cis*-acting sites have been shown to be necessary for transcriptional activation of several aflatoxin biosynthetic genes *in vivo* including *stcU* (Fernandes et al., 1998), *avnA* (Ehrlich et al., 2002), *pksA* (Cary et al., 2000) and *nor-1* (Miller et al., 2003a). AflR is necessary for transcriptional activation of several, if not all, aflatoxin biosynthetic genes.

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AflR - Regulation

Aflatoxin production is affected by environmental and nutritional factors such as pH, temperature, carbon source and nitrogen source (Miller et al., 2003c, Liu and Chu, 1998 and Luchese et al., 1993). Since AflR is a pathway regulator, the simplest model for these environmental and nutritional stimuli to impact aflatoxin gene transcription is directly through the *aflR* promoter. The timing of expression of *aflR* in response to these stimuli mimics the expression of aflatoxin structural genes (Miller et al., 2003c and Liu and Chu, 1998). *A. parasiticus* AreA (major nitrogen regulatory protein) can bind to the AflR promoter *in vitro* yet *in vivo* significance is unknown (Chang et al., 2000). A PacC (pH sensing) *cis*-acting site has also been identified in the *aflR* promoter using *in vitro* methods (Ehrlich et al., 1999a). A functional AflR binding site is located in the *aflR* promoter and is necessary for *aflR* transcription *in vivo* suggesting autoregulation (Ehrlich et al., 1999a). Additional studies on the transcriptional regulation of *aflR* are needed in order to understand how environmental, nutritional and even developmental stimuli affect aflatoxin biosynthesis.

Evidence for Other Transcription Factors

Though AflR is necessary for transcriptional activation of several, if not all, aflatoxin biosynthetic genes (Fernandes et al., 1998, Ehrlich et al., 2002, Cary et al., 2000 and Miller et al., 2003a), there is evidence for the involvement of additional transcription factors. Studies with the *pksA* promoter (Ehrlich et al., 2002) provided evidence that both PacC (pH sensing) and BrlA (sporulation) can impact *pksA* transcriptional regulation through *cis*-acting sites in the *pksA/nor-1* intergenic region (Figure 1.4). However, deletion of the consensus *cis*-acting sites for PacC and BrlA did not affect

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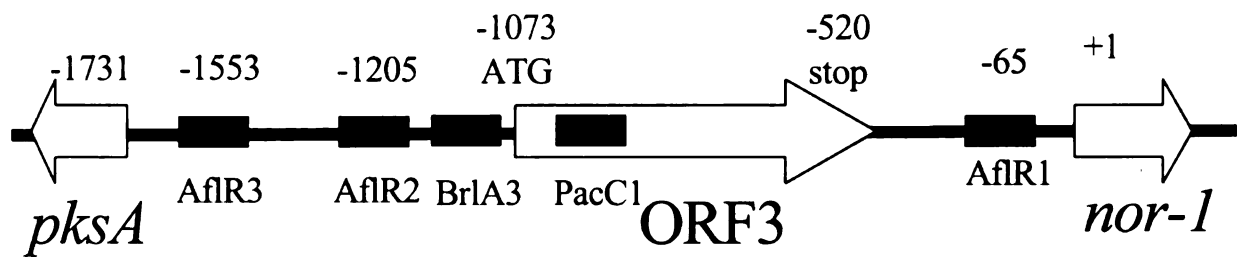


Figure 1.4. Schematic of the *nor-1/pksA* intergenic region in *A. parasiticus*. The numbers indicate the number of nucleotides included upstream from the primary transcriptional start site of *nor-1*. Several potential *cis*-acting sites are indicated including the AflR binding sites AflR1, AflR2 and AflR3 and PacC1 and BrlA3. The location of an open reading frame (ORF3) of unknown function is also shown. (Figure 1.4 adapted from Miller et al., 2003a)

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nor-1 transcriptional regulation in *A. parasiticus* under their culture conditions (Miller et al., 2003a).

The *A. nidulans* gene *stcU* (*A. parasiticus* *ver-1* homologue) also appears to be regulated by additional transcriptional factors besides AflR. A full length *stcU* promoter fused to a GUS reporter resulted in only a 2-3 fold increase in activity when transformed into a wild-type strain compared to transformation into an AflR mutant strain (Fernandes et al., 1998). In addition, substitution of both AflR binding sites only resulted in an approximately 5 fold reduction (not reduced to baseline expression levels) in GUS activity (Fernandes et al., 1998). Due to the relatively high activity in the AflR mutant strain and the moderate decrease resulting from binding site substitution, the possibility exists that other transcriptional activators besides AflR are involved in *stcU* transcriptional activation (Fernandes et al., 1998). For *avnA* in *A. parasiticus*, substitution of the AflR binding site resulted in a 10 fold decrease in promoter activity (Cary et al., 2000). While there is no evidence of other transcriptional activators in the *avnA* promoter, a potential *cis*-acting site for a repressor of aflatoxin biosynthesis was located (Cary et al., 2000). Deletion of 78 bp upstream of an AflR binding site in an *avnA*::GUS reporter construct resulted in a 3 fold increase in reporter activity (Cary et al., 2000). In addition, protein extracts collected under non-aflatoxin inducing condition demonstrated specific binding to this region (Cary et al., 2000).

The *nor-1* gene is perhaps the most studied of all the aflatoxin structural genes. A detailed analysis of the *nor-1* promoter has identified several *cis*-acting sites including AflR1, AflR2, a putative TATA box, CREbp and norLbp (Miller et al., 2003a and Miller et al., 2003b - Figure 1.4 and 1.5). Although putative TATA boxes have been identified in aflatoxin biosynthetic promoters, the *nor-1* TATA box was the first to be rigorously

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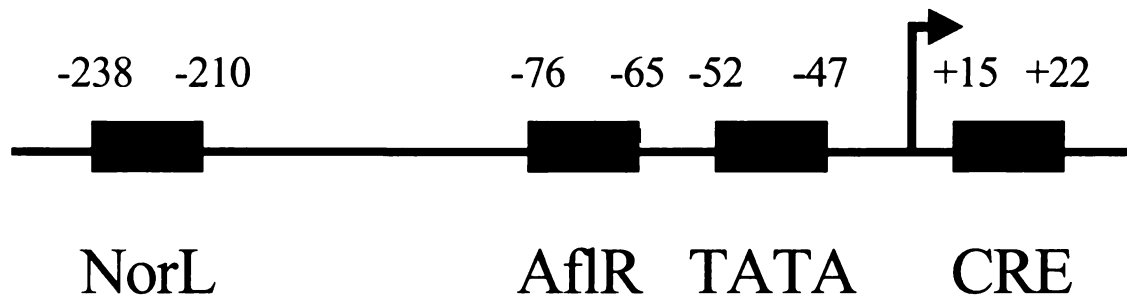


Figure 1.5. Identification of *cis*-acting sites in the *nor-1* promoter of *A. parasiticus*. The numbers indicate the number of nucleotides from the primary transcriptional start site of *nor-1*. (Figure 1.5 adapted from Miller et al., 2003b)

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tested for functional significance (Miller et al., 2003b). Substitution of the TATA box in the context of a larger promoter resulted in non-detectable GUS activity (Miller et al., 2003b). A novel *cis*-acting site (norL) was identified that is necessary for maximum *nor-1* transcriptional activation *in vivo*. Using electrophoretic mobility shift assays (EMSA), specific protein binding to norL was demonstrated (Miller et al., 2003b). EMSA also identified another potential *cis*-acting site, CRE1, in the *nor-1* promoter (Miller et al., 2003b and Miller et al., 2003c). Both the norL binding protein and CRE1 binding protein appear to rely on functional AfIR for maximum DNA binding (Miller et al., 2003b and Miller et al., 2003c). The transcriptional regulation of *nor-1* appears to involve additional proteins besides AfIR.

ORF3 and *nor-1/pksA* transcriptional activation

Reporter studies with *pksA::GUS* (Cary et al., 2000) and *nor-1::GUS* (Miller et al., 2003a) both demonstrated that inclusion of AfIR2 (Figure 1.4) resulted in greater transcriptional activation. We propose two alternative models to explain these data: 1) AfIR2 works synergistically with AfIR1 and AfIR3 to mediate transcription of the *nor-1* and *pksA* promoters respectively; or 2) AfIR2 mediates expression of ORF3 (potentially encodes a polypeptide of approximately 300 amino acid residues - Figure 1.4) directly downstream from AfIR2 which directly or indirectly impacts transcription of the *nor-1* and *pksA* promoters. Identification of a cDNA corresponding to ORF3 in an *A. parasiticus* cDNA library strongly suggests it represents a functional gene. Interestingly, blast searches using ORF3 as a query sequence have not provided solid clues regarding potential function. Model 2 allows us to make 2 related predictions regarding *nor-1* and *pksA* promoter function. 1) Since fungal isolates carrying *nor-1::GUS* or *pksA::GUS*

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constructs with promoter fragments that include a functional AflR2 and ORF3 show the highest GUS expression levels, accumulation of additional ORF3 protein in strains with two copies (native plus plasmid copy) overcomes a protein threshold resulting in extreme upregulation of *nor-1* and *pksA* promoter activity. 2) Loss of ORF3 function due to AflR2 deletion in the 2nd copy accounts for downregulation (or lack of upregulation) of both *nor-1* and *pksA* expression. Similar results are seen with the insertion of an additional copy of AflR (Chang et al., 1993, Chang et al., 1995 and Chang et al., 2001) suggesting a possible regulatory role for ORF3. Future experiments will determine the function of ORF3 and how AflR2 impacts *pksA* and *nor-1* transcriptional activation.

AflJ

The function of *aflJ* in aflatoxin biosynthesis is still unclear. The *aflJ* gene resides adjacent to *aflR* in the aflatoxin gene cluster with the two genes being divergently transcribed. An *A. flavus aflJ* knockout strain does not make aflatoxin and lacks the ability to convert several aflatoxin intermediates to aflatoxin (Meyers, et al., 1998). However, the *aflJ* knockout strain does accumulate several aflatoxin biosynthetic transcripts under aflatoxin conducive conditions suggesting that *aflJ* is not involved in the transcriptional regulation of aflatoxin biosynthesis (Meyers et al., 1998). Sequence analysis of *aflJ* did not reveal any enzymatic function but did identify three potential membrane-spanning domains and a putative microbody targeting signal (Meyers et al., 1998). Consequently, Meyers et al. proposed two hypotheses regarding AflJ function: 1) AflJ is involved in either transmembrane transport of aflatoxin pathway intermediates through intracellular compartments; or 2) AflJ is involved in the localization of pathway enzymes to an organelle (Meyers et al., 1998). Conversely, AflJ has been shown to

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interact with AflR in a two-hybrid assay (Chang and Yu, 2002). Insertion of an additional copy of *aflR* and *aflJ* into *A. parasiticus* resulted in greater aflatoxin biosynthesis than insertion of *aflR* alone while insertion of *aflJ* alone had no effect on aflatoxin biosynthesis (Chang et al., 2001). Consequently, Chang et al. have described *aflJ* as being a transcriptional co-activator (Chang et al., 2001). More work is needed to clearly define the activity of AflJ.

Regulation of Trichothecene Gene Transcription

Tri6 - Discovery

Following the identification of three trichothecene biosynthesis genes, *Tri5* (Hohn and Desjardins, 1992), *Tri4* (Hohn et al., 1995) and *Tri3* (McCormick et al., 1996), it was realized that they all resided in a 9-kb region and that the trichothecene biosynthesis genes might be clustered (Hohn et al., 1993b). The location of *aflR* within the aflatoxin biosynthetic gene cluster (Payne et al., 1993) suggested the possibility that other mycotoxin gene clusters, including trichothecene, may be regulated by a gene present within the cluster. While the discovery of *aflR* relied on complementation of a regulatory mutant (Payne et al., 1993), the initial identification of *Tri6* as a putative trichothecene biosynthesis transcriptional activator relied on additional sequencing of the trichothecene gene cluster (Proctor et al., 1995). Found immediately upstream of *Tri5*, *Tri6* is an open reading frame of 217 amino acids with regions similar to Cys₂His₂ zinc finger proteins (Proctor et al., 1995). The Cys₂His₂ zinc finger is a common motif for transcription factors including *BrlA* from *A. nidulans* (Adams et al., 1988).

Tri6 - Function

Several lines of evidence, in addition to sequence data, demonstrate that Tri6 is a transcriptional activator for trichothecene biosynthesis. *Tri6* expression mirrored the expression of other trichothecene genes including *Tri3* and *Tri4* (Proctor et al., 1995). Disruption of *Tri6* resulted in a strain with greatly reduced trichothecene production, trichothecene enzyme activities and trichothecene biosynthetic transcript steady-states (Proctor et al., 1995). Tri6 functioned as a transcriptional activator in *Saccharomyces cerevisiae* when fused to the DNA binding domain of Gal4 (Proctor et al., 1995). The Tri6 binding site was identified in *Fusarium sporotrichioides* as YNAGGCC using electrophoretic mobility shift assays with Tri6 produced *in vitro* (Hohn et al., 1999). The Tri6 binding site was confirmed *in vivo* using *F. sporotrichioides* Tri4 reporter strains (Hohn et al., 1999). All of the trichothecene biosynthetic genes identified in the *F. sporotrichioides* and *F. graminearum* clusters have the Tri6 binding site located in their promoter regions except for *Tri10* (Brown et al., 2001 and Hohn et al., 1999).

Tri10 - Discovery

Located upstream of *Tri5* in the trichothecene gene cluster (Figure 1.6), *Tri10* expression mirrored the timing of several trichothecene genes including *Tri6*, *Tri5* and *Tri4* (Tag et al., 2001). A *Tri10* disruption strain accumulated significantly less trichothecene biosynthetic transcripts (Tag et al., 2001). Conversely, transformants that had increased *Tri10* expression resulted in significantly increased trichothecene gene expression (Tag et al., 2001). As noted above, *Tri10* lacks a consensus Tri6 binding site (Brown et al., 2001) and is significantly upregulated in the *Tri6* disruptant strain (Tag et al., 2001). Tag et al. postulated that *Tri10* is not positively regulated by Tri6 but instead

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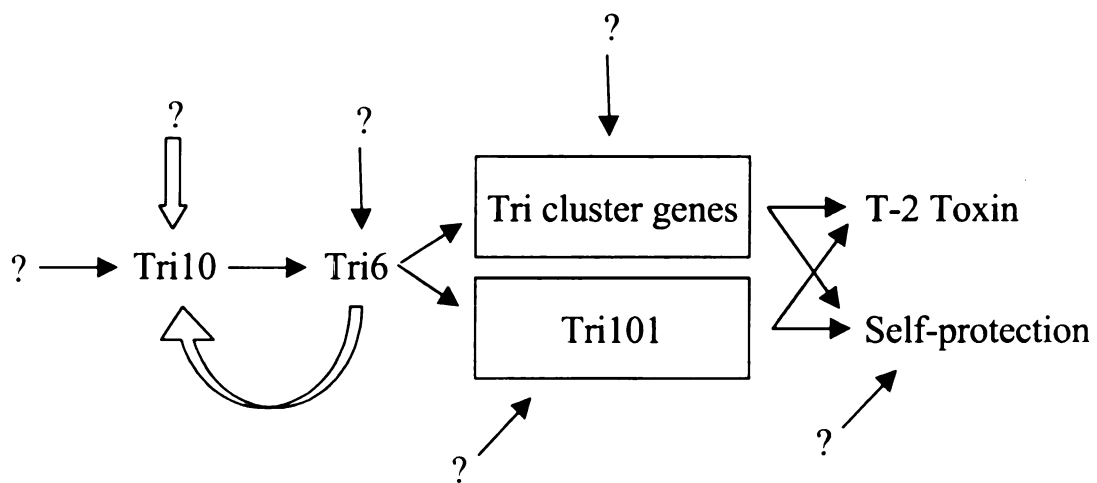


Figure 1.6. Proposed regulatory model for trichothecene biosynthesis. Solid arrows indicate positive activators while open arrows indicate inhibitory activities. Question marks indicate other proposed but unknown regulatory signals or factors. (Figure 1.6 adapted from Tag et al., 2001)

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may be negatively regulated by unknown mechanisms when Tri6 is present (Tag et al., 2001). The impact of Tri10 extends to genes involved in the primary metabolic steps that generate trichothecene precursors including *Fpps* (Tag et al., 2001). While Tri10 lacks any known transcription factor motif, its function is clear as an essential regulator in trichothecene gene regulation (Tag et al., 2001). Future investigations are focused on determining the precise mode of action of Tri10 and on further identification of the regulatory circuits defined by Tri10 (Tag et al., 2001).

Regulation of Biosynthesis Gene Transcription for other Mycotoxins

Although most information regarding mycotoxin gene regulation is centered on aflatoxin and trichothecene biosynthesis, other mycotoxin related transcription factors have been identified. In fact, information about aflatoxin and trichothecene biosynthesis pathways have aided in the analysis of different mycotoxin pathways. For example, a Tri6 homolog has been identified (MRTri6) in the distantly related trichothecene pathway of *Myrothecium roridum* (Hohn et al., 1999). *Dothistroma pini* synthesizes dothistromin, a difuranoanthraquinone toxin similar to aflatoxin, with several analogous genes (Bradshaw et al., 2002). Bradshaw et al. hope to potentially identify the dothistromin pathway regulator by using *A. parasiticus aflR* as a probe and/or by sequencing the entire dothistromin gene cluster (Bradshaw et al., 2002). Sequencing of the paxilline biosynthesis cluster in *Penicillium paxilli* found two potential transcription factors, *paxR* and *paxS* (Young et al., 2001). Like *aflR*, both *paxR* and *paxS* contain the uniquely fungal Zn(II)₂Cys₆ binuclear cluster DNA binding motifs (Young et al., 2001). It is currently unknown the exact function of *paxR* and *paxS* in paxilline biosynthesis.

Mycotoxin Gene Clusters

While common in prokaryotes, the linkage of functionally related genes is relatively rare in higher eukaryotes. In filamentous fungi, however, the clustering of genes is a common feature for several metabolic pathways. Examples of gene clusters in secondary metabolism include aflatoxin (Trail et al., 1995a), trichothecene (Brown et al., 2001), penicillin (Diez et al., 1990), fumonisin (Seo et al., 2001), dothistromin (Bradshaw et al., 2002), paxilline (Young et al., 2001) and several others. Several nutrient utilization pathways are also clustered in filamentous fungi including ethanol (Fillinger and Felenbok, 1996) and nitrate (Johnstone et al., 1990) utilization in *A. nidulans*. The physical linkage of related genes suggests two possible hypotheses: 1) linkage of metabolic pathway genes provides a means to regulate pathway gene transcription; and 2) linkage provides a means for genetic transfer of entire pathways between species. Both of these hypotheses are discussed below.

Organization of Gene Clusters

Aflatoxin Gene Cluster

Analysis of aflatoxin pathway mutants first established the likely clustering of at least some of the aflatoxin biosynthetic genes (reviewed in Bennett and Papa, 1988). Subsequently, the first two aflatoxin genes identified, *nor-1* and *ver-1*, were found to localize to the same *A. parasiticus* cosmid (Trail et al., 1995b). The aflatoxin gene cluster has been mapped in *A. parasiticus* (Trail et al., 1995b), *A. flavus* (Yu et al., 1995a) and *A. nidulans* (Brown et al., 1996). The timing of transcript accumulation of all the cluster genes is consistent with their involvement in aflatoxin biosynthesis (Trail et al., 1995b and Brown et al., 1996). In fact, many of the genes have been studied in detail

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and been shown to be involved in aflatoxin biosynthesis. Interestingly, the aflatoxin gene clusters of these three related organisms are not organized identically. For example, the distance between *aflR* and *ver-1* is 32 kb in *A. nidulans* but 8 kb in *A. parasiticus* and *A. flavus*. The maintenance of gene clusters despite changes in gene order suggests that gene clustering has functional significance for the fungi.

A 5 kb spacer region is located at one end of the aflatoxin gene cluster (Yu et al., 2000a and Yu et al., 2000b). The spacer region contains no open reading frames (ORFs) and has a sugar utilization cluster located on the other side of the spacer region (Figure 1.7 – Yu et al., 2000a and Yu et al., 2000b). Aflatoxin production is closely linked to carbon source with simple sugars like glucose and sucrose able to induce aflatoxin biosynthesis (Buchanan and Lewis, 1984). Consequently, the localization of this sugar utilization cluster near the aflatoxin cluster suggests a regulatory connection between the two clusters. However, of the four genes in the sugar cluster, only *hxtA* (proposed hexose transporter protein) expression was shown to be concurrent with aflatoxin pathway cluster genes (Yu et al., 2000a). It is unknown if there are any functional *AflR cis*-acting sites in the sugar cluster gene promoters. In addition, the border at the other end of the aflatoxin gene cluster has not yet been defined.

Trichothecene Gene Cluster

Two overlapping cosmid clones were able to complement different trichothecene mutants suggesting that the trichothecene genes were clustered in *Fusarium sporotrichioides* (Hohn et al., 1993b). A 23 kb trichothecene gene cluster has been sequenced for both *F. sporotrichioides* and *F. graminearum* and contains 12 genes (Brown et al., 2001). All of the 12 clustered genes studied so far have been shown to be

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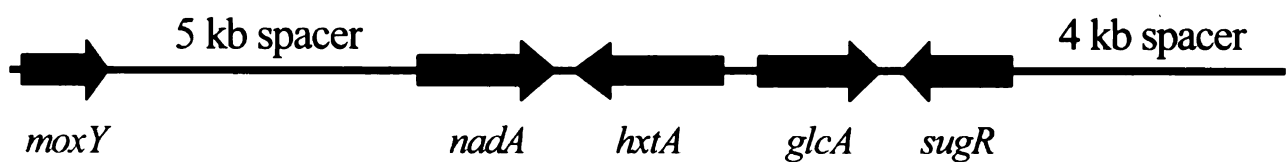


Figure 1.7. Schematic of the sugar utilization gene cluster in *A. parasiticus*. The *moxY* gene is at one end of the aflatoxin gene cluster. The spacer regions on either side of the sugar utilization cluster do not contain open reading frames. Map is roughly to scale.

(Figure 1.7 adapted from Yu et al., 2000a)

involved in trichothecene biosynthesis (Figure 1.8) (reviewed in Brown et al., 2001). Yet, the 12 genes in the identified trichothecene gene cluster are insufficient to account for all known trichothecene structures (Brown et al., 2001) leading to two hypotheses: 1) additional trichothecene biosynthesis genes are located beyond the flanking sequence of *tri8* and *tri12*; and 2) all trichothecene genes are not located within the gene cluster. Since most, if not all, of the genes necessary for aflatoxin biosynthesis are located within the aflatoxin gene cluster, it would be reasonable to sequence beyond *tri8* and *tri12* to potentially identify additional trichothecene genes. In addition, it will be interesting to determine if there is an extended spacer region that forms a border of the trichothecene gene cluster as there is with the aflatoxin gene cluster.

tri101 exists outside of the 23 kb cluster in both *F. sporotrichioides* (McCormick et al., 1999) and *F. graminearum* (Kimura et al., 1998a) and is the only known trichothecene gene to exist outside of the cluster. The genes on either side of *tri101* (UTP-ammonia ligase and phosphate permease) are not involved in trichothecene biosynthesis and *tri101* is at least 35 kb from either end of the identified trichothecene cluster in *F. graminearum* (Kimura et al., 1998b). Tri101 is a 3-O-acetyltransferase that is required for T-2 production in *F. sporotrichioides* (McCormick et al., 1999). Since the enzymatic products of Tri101 are less toxic than the substrates, it was originally proposed that the purpose of Tri101 was to protect the fungus (Kimura et al., 1998a). Expression of *tri101* in yeast (Kimura et al., 1998a and McCormick et al., 1999) and plants (Muhitch et al., 2000) resulted in increased tolerance for trichothecenes. Yet, a *F. sporotrichioides* *tri101* disruptant could both germinate and grow in the presence of trichothecenes suggesting that *tri101* is not an essential self-defense mechanism for *F. sporotrichioides* (McCormick et al., 1999). The evolution of *tri101* is discussed in later sections.

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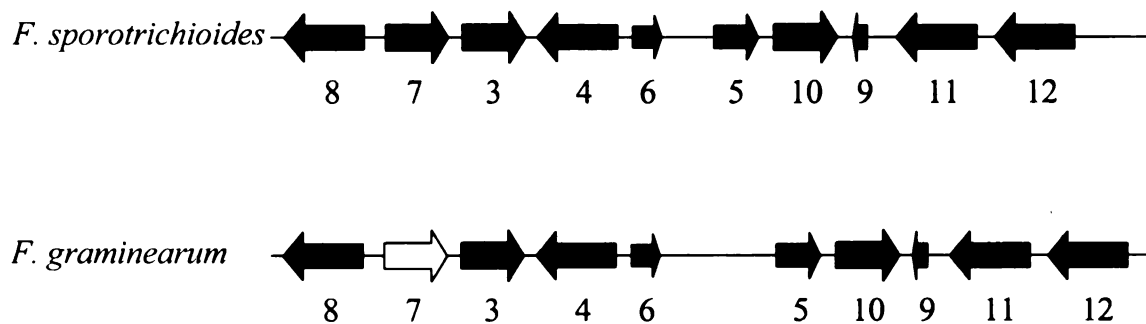


Figure 1.8. Genomic organization of the trichothecene biosynthetic gene cluster of *Fusarium sporotrichioides* and *F. graminearum*. Arrowheads indicate the direction of transcription and the number underneath each arrow refer to the specific gene. Genes with the same number from both *Fusarium* species are homologues. *tri7* in *F. graminearum* is non-functional. Map is roughly to scale. (Figure 1.8 adapted from Brown et al., 2001)

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Fumonisin Gene Cluster

The identification of the fumonisin gene cluster utilized allele tests with three different *Fusarium moniliforme* fumonisin mutants (Desjardins et al., 1996). Subsequently, a polyketide synthase gene, *fum5*, was isolated and shown to be required for fumonisin biosynthesis (Proctor et al., 1999). Based on the structure of fumonisin B1, at least 8 different enzymatic steps are needed for fumonisin biosynthesis (Seo et al., 2001). Sequencing downstream from *fum5* identified four additional ORFs, *fum6*, -7, -8 and -9, whose expression is correlated with fumonisin production (Figure 1.9 – Seo et al., 2001). Gene disruption analysis of *fum6* and *fum8* revealed that they are necessary for fumonisin biosynthesis (Seo et al., 2001). Nucleotide sequence analysis in the DNA regions flanking *fum5* and *fum9* may identify additional fumonisin biosynthetic genes. Based on aflatoxin and trichothecene gene clusters, it is reasonable to expect that a transcriptional activator specific for fumonisin biosynthesis located within the fumonisin gene cluster will be found with the additional sequencing.

Impacts of Mycotoxin Gene Clusters

Identification of Gene-Cluster-Dependent Regulation

Preliminary evidence for a role for clustering in aflatoxin gene regulation was reported by Liang et al. (Liang et al., 1997). The promoter of the aflatoxin biosynthesis structural gene *ver-1* was fused to *uidA* (encodes β -glucuronidase [GUS]) to generate a reporter plasmid (pHD6.6) that contained *niaD* (encodes nitrate reductase) as a selectable marker (Liang et al., 1997). pHD6.6 integrated predominantly at the *ver-1* or *niaD* locus via homologous recombination (Liang et al., 1997). Single-copy integration of pHD6.6 at *niaD* resulted in a 500-fold reduction in *ver-1* promoter activity when compared with

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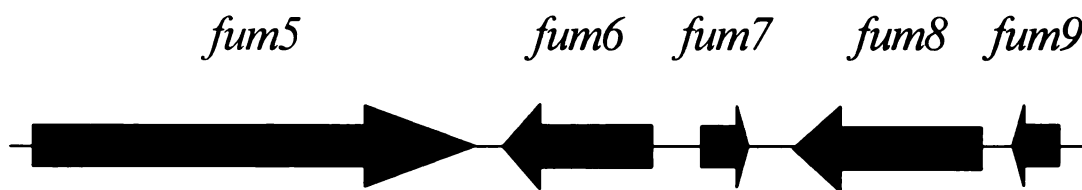


Figure 1.9. Schematic of the fumonisin biosynthetic gene cluster in *Fusarium verticillioides*. Arrowheads indicate the direction of transcription. Map is roughly to scale. (Figure 1.9 adapted from Seo et al., 2001)

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single-copy integration at the *ver-1* locus; however, the temporal pattern of expression appeared to be similar at both loci (Liang et al., 1997). One explanation for reduced *ver-1* promoter function at the *niaD* locus is that location in the aflatoxin cluster results in positive, position-dependent regulation of *ver-1* expression. An alternative hypothesis is that the expression of *ver-1* integrated at *niaD* is negatively influenced by *niaD* regulation. In the absence of preferred nitrogen sources and in the presence of nitrate, *niaD* is expressed. Under the rich growth conditions tested by Liang et al. (Liang et al., 1997), *niaD* may have been repressed, and therefore the lack of *ver-1* expression at the *niaD* site could have resulted from *niaD*-dependent regulation.

Subsequently, the promoter of the aflatoxin biosynthesis gene *nor-1* was fused to GUS to generate a reporter plasmid, pAPGUSNN-B, containing *niaD* as a selectable marker (Chiou et al., 2002). Transformants with pAPGUSNN-B integrated at the *niaD* locus had no detectable GUS activity while *nor-1* integrants had GUS activity (Chiou et al., 2002). In addition, the cloned *nor-1* promoter functioned similarly to the native *nor-1* promoter when it integrated at the *nor-1* locus (Chiou et al., 2002). Because *niaD* dependent regulation could account for the absence of expression at *niaD* for both *nor-1::GUS* (Chiou et al., 2002) and *ver-1::GUS* (Liang et al., 1997), a third chromosomal location was analyzed using pAPGUSNP, which contained *nor-1::GUS* plus *pyrG* (encodes OMP decarboxylase) as a selectable marker (Chiou et al., 2002). GUS expression was detectable only when pAPGUSNP integrated at *nor-1* and was not detectable at *pyrG*, even under growth conditions that required *pyrG* expression (Chiou et al., 2002). While the mechanism is unknown, *nor-1* and perhaps other aflatoxin biosynthetic genes are susceptible to aflatoxin gene-cluster-dependent regulation.

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The genome of some strains of *A. parasiticus* includes a partial duplication of the aflatoxin gene cluster (Chang and Yu, 2002). The region from *aflR* to *ver-1* plus *omtB* is duplicated in *A. parasiticus* SU1 (Chang and Yu, 2002). It is unknown why the genes between *ver-1* and *omtB* are not in the duplicated region. All of the duplicated genes appear to have mutations in them that would make them non-functional except for *aflR-2* and *aflJ-2* (Chang and Yu, 2002). Northern and RT-PCR analyses of RNA indicated that *aflR-2* is expressed at much lower levels than *aflR-1* (Cary et al., 2002). Nucleotide sequence analysis upstream of the *aflR-2* translational start codon revealed that the AflR binding site was intact and that there were few base changes (2%) compared to the corresponding region of *aflR-1* (Cary et al., 2002). Cluster-dependent regulation may explain the poor expression of *aflR-2* (Cary et al., 2002 and Chang and Yu, 2002). It is currently unclear if the other genes in the duplicated region are expressed at similar levels to their cluster counterparts. Reporter constructs that can integrate into the aflatoxin gene cluster and the duplicated region may provide valuable insight into the mechanism of cluster-dependent regulation.

Trichothecene biosynthesis genes are also clustered (Hohn et al., 1993b) and there is some evidence for position-dependent regulation with the pathway regulator *tri6* (Chen et al., 2000). Introduction of a plasmid containing a *tri5::GUS* fusion with a functional *tri6* resulted in 50 to 100 fold more GUS activity with *tri5* integration compared to ectopic integration (Chen et al., 2000). However, there were no differences in GUS activity between *tri5* and ectopic integration of a *tri5::GUS* plasmid without a functional *tri6* (Chen et al., 2000). Integration of a *tri4::GUS* fusion into the *tri4* locus resulted in 2 to 5 fold more GUS activity than ectopic integration (Hohn et al., 1999). Additional

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experiments are necessary to clarify the significance of cluster-dependent regulation in trichothecene biosynthesis.

It is currently unknown if cluster-dependent regulation occurs with other mycotoxin gene clusters like fumonisin. However, cluster-dependent regulation appears to be less significant with trichothecene biosynthesis than with aflatoxin biosynthesis. Except for aflatoxin biosynthesis, the description of mycotoxin gene-cluster-dependent regulation needs further study. While the construction of plasmids with mycotoxin promoters fused to reporter genes is useful in identifying *cis*-acting sites in the mycotoxin promoter, they also provide a means to determine differences between mycotoxin cluster integration and ectopic integration.

Significance of Gene-Cluster-Dependent Regulation

The targeting of reporter constructs to specific chromosomal locations for promoter analysis has been suggested by other investigators studying expression of fungal genes (Hamer and Timberlake, 1987 and Timberlake and Marshal, 1988 and van Gorcom et al., 1986). The gene-cluster-dependent regulation seen with the aflatoxin gene cluster emphasizes the importance of determining the site of integration for mycotoxin reporter plasmids. In addition, screening transformants by genotype (site of integration) rather than phenotype (reporter activity) will prevent possible sampling bias. A rapid method for screening the site of integration for *A. parasiticus* has been described (Chiou et al., 2002) that makes screening by genotype feasible.

Although the mechanisms of position-dependent gene expression have not been fully elucidated in filamentous fungi, it has been hypothesized that enhancer elements may be responsible for the position-dependent effect (Kinsey and Rambosek, 1984).

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Studies performed on the SpoC1 gene cluster in *A. nidulans* showed that clustered genes can be coordinately expressed during development and that placement of cluster genes at ectopic chromosomal locations results in the loss of that coordination (Miller et al., 1987 and Timberlake and Barnard, 1981). The hypothesis that positive *cis*-acting factors have regional control over the transcription of aflatoxin genes is reasonable, since removal of aflatoxin reporter fusions from the aflatoxin gene cluster results in reduced GUS expression.

More recently, studies with the mammalian β -globin gene identified a region named a locus control region (LCR) (Grosveld et al., 1987). The β -globin LCR was identified as a region that was necessary to confer positional independence of a β -globin transgene (Grosveld et al., 1987). Subsequently, several additional LCRs have been identified (reviewed in Li et al., 2002). We hypothesize that aflatoxin gene expression is influenced by a locus control region. However, a locus control region has not been identified in fungal gene clusters. If a LCR does influence the regulation of *nor-1* transcription, it is located at least 3 kb upstream from the transcription initiation site in the 5' *nor-1* region, at least 1.8 kb downstream of the transcription termination site in the 3' region of *nor-1*, or within the *nor-1* coding region. Because similar position-dependent expression is observed with the *ver-1::GUS* reporter construct (Liang et al., 1997), it is possible that the same LCR element is influencing the regulation of both *nor-1* and *ver-1*. Comparison of steady state levels of mRNA transcripts from genes present in the aflatoxin gene cluster and the duplicated region (ie *ver-1A* vs. *ver-1B*) may help narrow the search for the LCR(s). It will be interesting to determine if the duplicated region includes the LCR(s) necessary for cluster-dependent regulation. In addition, the 5 kb spacer region at one border of the aflatoxin gene cluster is a tempting place to look for

possible boundary elements. Boundary elements appear to provide a functional boundary for both accessible and inaccessible chromatin (Labrador and Corces, 2002). The possible boundary element in the spacer region would prevent the LCRs from affecting transcriptional activation of genes in the sugar cluster or beyond.

While solid evidence for cluster-dependent regulation has only been shown for aflatoxin biosynthesis, it is reasonable to predict that several other mycotoxin gene clusters will also be subject to cluster-dependent regulation. Consequently, the proper use of reporter plasmids to study mycotoxin gene transcriptional regulation must include the identification of the site of integration of the plasmids to ensure proper data interpretation.

Evolution of Mycotoxin Gene Clusters

Origin of the Aflatoxin Gene Cluster

The occurrence of fungal pathway gene clusters may result from horizontal gene transfer from prokaryotes where clustering of pathway genes is common (Hohn and Keller, 1997). Perhaps the strongest case for the horizontal gene transfer of an entire secondary metabolic pathway from bacteria to fungi is with penicillin biosynthesis (Aharonwitz et al., 1992 and Weigel et al., 1988). The G+C content of the penicillin gene cluster is more similar to *Streptomyces* than the producing fungi and the fungal penicillin genes lack introns which is generally a characteristic of bacteria (Hohn and Keller, 1997). Horizontal gene transfer is unlikely with the aflatoxin biosynthetic pathway due to the presence of introns and similar G+C content inside and outside of the aflatoxin cluster (Brown et al., 1996). Yet the changes in gene order within the aflatoxin gene cluster of different *Aspergilli* suggests that there is selective pressure to keep the

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aflatoxin genes clustered. *Dothistroma pini*, a fungal pine pathogen, produces the mycotoxin dothistromin that is structurally similar to versicolorin A, an intermediate in aflatoxin biosynthesis (Bradshaw et al., 2002). Four genes identified in the *D. pini* dothistromin gene cluster all have high similarity with known aflatoxin biosynthesis genes (Bradshaw et al., 2002). Detailed analysis of the dothistromin and aflatoxin gene clusters may help explain the evolutionary history of the aflatoxin gene cluster.

Origin and Diversity of Trichothecene Gene Clusters

The current literature provides few clues for the origin of the trichothecene gene cluster. The ten trichothecene genes identified so far in the cluster (Tri-3 through Tri-12) contain a total of 37 introns (Brown et al., 2001) suggesting that horizontal gene transfer from prokaryotes is unlikely. While there are many different trichothecenes that are produced by various fungi, *Fusarium* species appear to be the primary source of trichothecenes in agricultural products (Hohn et al., 1999). The mechanism for how different *Fusarium* species produce different trichothecenes is unknown. The T-2 gene cluster in *Fusarium sporotrichioides* and the deoxynivalenol gene cluster in *F. graminearum* are very similar (see Figure 1.8) in that the 23 kb region included 12 homologous genes (Brown et al., 2001). However, Tri7 (acetylates the oxygen on C-4) is non-functional in *F. graminearum* (Brown et al., 2001) suggesting a possible mechanism for generating trichothecene structural diversity. The origin of the non-cluster *tri101* gene may be different than the clustered trichothecene genes (Kimura et al., 1998c). Kimura et al. reasoned that a translocation event was unlikely to explain the location of *tri101* because it is flanked by essential primary metabolism genes (Kimura et al., 1998b). In addition, homologues of *tri101* were found in *Saccharomyces cerevisiae* and

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Schizosaccharomyces pombe (Kimura et al., 1998b). Consequently, Kimura et al. reasoned that it is feasible that trichothecene producers have acquired *tri101* through horizontal gene transfer (Kimura et al., 1998b).

ACKNOWLEDGMENTS

Two sections from Chapter 1 (Transcriptional Regulation of Mycotoxin Biosynthesis and Mycotoxin Gene Clusters) will appear in the chapter titled “Fungal Genetics and Mycotoxin Biosynthesis” in Food Biotechnology. The chapter will be co-authored with Dr. John Linz who has written other sections of the chapter. The publication date for Food Biotechnology hasn't been set but we expect 2004.

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CHAPTER 2

Effects of nitrogen source, carbon source and zinc concentration on the regulation of the aflatoxin biosynthetic gene *nor-1* from *Aspergillus parasiticus*

INTRODUCTION

Aflatoxins are highly toxic and carcinogenic secondary metabolites of certain strains of *Aspergillus parasiticus*, *A. flavus* and *A. nomius* (Wilson and Payne, 1994). Several different crops have been found to be contaminated with aflatoxins including peanuts, cottonseed, corn, pistachios, walnuts, and almonds (Dvorackova, 1990). Epidemiological data have linked aflatoxins with human hepatic cancer, primarily in countries where the elimination of contaminated products is not practiced (Reviewed in Dvorackova, 1990 and Hall and Wild, 1994). Aflatoxin carcinogenicity studies clearly demonstrate its high potency in various laboratory animals (reviewed in World Health Organization, 1979). Due to the animal and human toxicology data, aflatoxin susceptible crops are monitored for aflatoxin contamination in the United States with an action level of 20 ppb for food for human consumption, 0.5 ppb for milk and 20-300 ppb for most animal feeds (Council for Agricultural Science and Technology, 2003). Due to health and economic concerns, the elimination of aflatoxin from the food chain is desirable.

One approach to achieve the goal of aflatoxin elimination has been to elucidate the biosynthesis of aflatoxin at the molecular level. Aflatoxin biosynthesis is a complex process that requires at least 16 different enzymatic steps (Bhatnagar et al., 1994). The aflatoxin biosynthesis genes reside in a 70 kb cluster and appear to be co-regulated (Trail

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et al., 1995b). Located within the aflatoxin gene cluster are the genes *nor-1*, *ver-1* and *aflR*. *Nor-1* catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin (Zhou and Linz, 1999). The *nor-1* gene is physically located between *pksA* and *fas2*, two of the first three (*fas1* is the other) structural genes in aflatoxin biosynthesis (Trail et al., 1995b). *Ver-1* is associated with the conversion of versicolorin A to sterigmatocystin, a late step in aflatoxin biosynthesis (Skory et al., 1992). The *ver-1* gene is physically located at the other end of the aflatoxin gene cluster from *nor-1*. Analysis of *nor-1* and *ver-1* transcriptional activation provides a good cross-section for the aflatoxin biosynthesis structural genes.

The accumulation of *nor-1* and *ver-1* transcripts were shown to be coregulated (Skory et al., 1993) along with several other aflatoxin biosynthesis genes (Trail et al., 1995b). Analysis of the regulatory mutant *A. flavus* strain 650 (Bennett and Papa, 1988) first identified *aflR* as a pathway regulator (Payne et al., 1993). Subsequently, *aflR* has been identified in *A. parasiticus* (Chang et al., 1993) and *A. nidulans* (Yu et al., 1996). While *AflR* has been clearly implicated in the regulation of specific aflatoxin biosynthetic genes (Cary et al., 2000, Fernandes et al., 1998, Ehrlich et al., 2002 and Miller et al., 2003a), it is unclear whether *AflR* alone is sufficient to stimulate transcription of all aflatoxin biosynthetic genes.

Environmental and nutritional factors such as temperature, pH, carbon source, nitrogen source and zinc concentration all strongly influence aflatoxin accumulation in laboratory media (reviewed in Luchese and Harrigan, 1993). Defined media such as Reddy's, GMS (glucose minimal salts) and PMS (peptone minimal salts, semi-defined) were designed to investigate the effects of metals, nitrogen source and carbon source on aflatoxin production. Several researchers have demonstrated that glucose as a sole

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carbon source supports aflatoxin production while peptone does not (Buchanan and Stahl, 1984, Abdollahi and Buchanan, 1981a and Abdollahi and Buchanan, 1981b). Nutritional shift experiments with transcriptional and translational inhibitors indicated that glucose stimulates *de novo* synthesis of aflatoxin biosynthesis transcripts and proteins (Abdollahi and Buchanan, 1981b). Various nitrogen sources including amino acids, nitrate and ammonium have been extensively studied in *Aspergillus parasiticus* growth and aflatoxin formation (reviewed in Luchese and Harrigan, 1993). While reports vary with what nitrogen source results in the most aflatoxin production, it is clear that ammonium is significantly more stimulatory than nitrate with ammonium nitrate (NH_4NO_3) intermediate. Nitrate as the sole nitrogen source has been shown to inhibit *de novo* synthesis of aflatoxin biosynthesis enzymes (Kachholz and Demain, 1983). Metal ions also can affect aflatoxin biosynthesis as well. In particular, omission of zinc in the culture medium results in non-detectable levels of aflatoxin (Coupland and Niehaus, 1987 and Bennett et al., 1979). In addition, researchers have shown a correlation between zinc concentration in corn and their aflatoxin concentration (Failla et al., 1986). Nutritional factors such as carbon source, nitrogen source and zinc concentration provide a useful tool in studying the molecular mechanisms of aflatoxin biosynthesis gene regulation.

Our hypothesis is that these nutritional factors affect transcriptional regulation of aflatoxin biosynthetic genes. The purpose of this study was to use nutritional factors to study the regulation of the aflatoxin biosynthesis genes *nor-1*, *ver-1* and *aflR* in batch cultures using the *nor-1::GUS* reporter strain D8D3 and the *ver-1::GUS* reporter strain 14. The *nor-1::GUS* and *ver-1::GUS* strains permit the study of transcriptional activation of *nor-1* and *ver-1*, respectively, in these various media. GMS (glucose minimal salts)

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supported aflatoxin biosynthesis, *nor-1*, *ver-1*, and *aflR* transcript accumulation, Nor-1 and Ver-1 protein accumulation and *nor-1::GUS* and *ver-1::GUS* activity. PMS (bacto-peptone minimal salts - D8D3 only) and NMS (glucose minimal salts with nitrate), supported low to non-detectable aflatoxin biosynthesis, *nor-1*, *ver-1*, and *aflR* transcript accumulation, Nor-1 and Ver-1 protein accumulation and *nor-1::GUS* and *ver-1::GUS* activity. Aflatoxin biosynthesis with GMS_0 (glucose minimal salts with no Zinc added) was similar to *GMS* (glucose minimal salts) at 24 hours but aflatoxin production, transcript accumulation, protein accumulation and GUS activity decreased thereafter. Using a 160 base pair *nor-1* promoter fragment as probe (*nor-R*), electrophoretic mobility shift assays (EMSA) with *A. parasiticus* SU1 (wild type) and AFS10 (*AflR* knock-out) PMS extracts both contained one specific complex of similar mobility that did not bind to the *AflR* binding site (TCGnnnnnCGA) that is included in *nor-R*. *GMS* protein extracts from *A. parasiticus* AFS10 produced no specific shifted complexes with *nor-R*. *A. parasiticus* SU1 *GMS* protein extracts produce a specific complex with *nor-R* (different mobility than the specific shifts seen with the PMS extracts) that also does not involve the *AflR* binding site. Competition experiments localized the potential *cis*-acting site in *nor-R* to a region designated CRE1. This work is a comprehensive analysis of the use of nutritional components as tools to study aflatoxin gene transcriptional regulation.

MATERIALS AND METHODS

Strains and Growth Conditions

A. parasiticus D8D3 contains a *nor-1::GUS* (*GUS* = *uidA* from *Escherichia coli*) translational fusion at the 3' region of *nor-1* (Chiou et al., 2002). *A. parasiticus* Isolate 4

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(I4) contains the *ver-1::GUS* translational fusion at the 3' region of *ver-1* (Liang et al., 1997). *A. parasiticus* SU1 is a wild type aflatoxin producing strain. *A. parasiticus* AFS10 is an *aflR* knock-out strain (Cary et al., 2002).

GMS and PMS media were made as described by Buchanan with the exception of the changes described in Table 2.1 (Buchanan and Lewis, 1984). For liquid culture experiments, 100 mL of medium in a 250 mL flask with 5, 6-mm, glass beads were inoculated with 2×10^6 spores and incubated at 29°C in the dark with shaking at 150 rpm. D8D3 and I4 were grown in triplicate for each time point. The time points for D8D3 were 36, 48 and 72 h. I4 grows slower than D8D3 and there was not enough mycelia for analysis at 36 h. Consequently, the time points for I4 were 48, 60 and 72 h. YES medium (2% yeast extract, 6% sucrose; pH=5.8) was used for Nor-1:Maltose binding protein studies.

Table 2.1. Medium components used in this study.

medium ^a	carbon source	nitrogen source	zinc concentration
PMS	peptone	ammonium (+ peptone)	10 mm
NMS	glucose	nitrate	10 mm
GMS ₀	glucose	ammonium	0 mm
GMS	glucose	ammonium	10 mm

^a Base medium was the same for all as previously described (Buchanan and Lewis, 1984).

RNA and Protein Extraction

Liquid cultures of *A. parasiticus* D8D3 and I4 were filtered at the indicated time points through miracloth (Calbiochem, La Jolla CA), the mycelia was then frozen with liquid nitrogen and stored at -80°C. For RNA extraction, approximately 200 mg of

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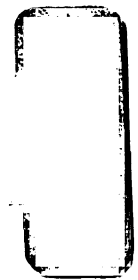
frozen mycelia was macerated with a mortar and pestle. TriZOL (Gibco BRL, Grand Island NY) was used to extract total RNA from the ground mycelia following manufacturers instructions. For protein extraction, approximately 200 mg of frozen mycelia was macerated with a mortar and pestle. The ground mycelia was suspended in 0.5 ml of GUS lysis buffer (50 mM NaH₂PO₄, 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 10 mM β-mercaptoethanol, pH 7.0), vortexed for 15 seconds and centrifuged for 10 minutes at 10,000 X g. The supernatent was carefully removed and placed in a new tube and stored on ice. The protein concentration was determined using the BioRad (BioRad, Hercules CA) protein dye reagent following manufacturer's instructions.

Northern Hybridization Analysis

Northern analysis was performed as described in Current Protocols in Molecular Biology (Ausubel et al., 2003) with 15 µg of total RNA loaded per lane and electrophoresed in a 1% agarose gel with 0.4 M formaldehyde. The resolved RNA was transferred by capillary action to a Nytran nylon membrane (Schleicher & Schuell, Keene NH) and immobilized by UV crosslinking in a Stratalinker (Stratagene, La Jolla CA). *nor-1* and *ver-1* DNA probes were generated by PCR using the *nor-1* cDNA plasmid pQE31 (Zhou and Linz, 1999) and the cosmid *norA* (Trail et al., 1995b) respectively. The primer pairs for each reaction were: *nor-1*, 5'-GCGACACGAACCCAG-3', 3'-CGTCCAAAACGACC-5'; *ver-1*, 5'-AGCGCGGAGCCAAAG-3', 3'-CGGGCGACATCCACAG-5'. The PCR products were gel purified using the QiaexII Gel Extraction Kit (Qiagen, Santa Clarita CA). Approximately 120 ng of each DNA fragment was radio labeled by the random primed method (BMB, Indianapolis IN) using 50 µCi [³²P]-dCTP (NEN, Boston MA). The probes were hybridized to the immobilized RNA for 16 hours

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at 65°C. The membranes were washed twice at room temperature for 15 minutes under low stringency (2X SSC, 0.1% SDS) followed by a high stringency wash at 65°C (0.2X SSC, 0.1% SDS). Signals were detected using a Phosphorimager FX (BioRad, Hercules CA).

Western Blot Analysis

Western blot analyses were performed by standard procedures (Ausebel et al., 2003). Thirty µg of total protein were resolved by electrophoresis through a 12% acrylamide gel using a Miniprotean II electrophoresis cell (BioRad, Hercules CA). The proteins were electroblotted to PVDF membrane and probed with anti-Nor-1 or anti-Ver-1 (Liang et al., 1997) as primary antibody. Alkaline phosphatase labeled rabbit anti-IgG (Sigma, St. Louis MO) was used as a secondary antibody. Colorimetric detection of the enzyme linked secondary antibody was carried out using BCIP/NBT tablets (Amresco, Solon OH). Duplicate gels were stained with Coomassie Brilliant Blue R-250 to evaluate the composition and condition of the protein extracts and to verify equal loading.

Liquid Culture GUS Assays

Liquid culture GUS assays were performed with 1 mg of fresh protein extract as described by Miller (Miller et al., 2003a). GUS activities are reported as the mean of the triplicate flasks, analyzed twice with the standard deviation represented by errors bars.

Protein Extraction for EMSA

Cellular protein was extracted from cultures of *A. parasiticus* SU1 using modifications of the methods of Peters and Perez-Esteban (Peters and Caddick, 1994 and

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Perez-Esteban et al., 1993). 1 liter flasks containing 10, 6mm glass beads and 500 mL medium (GMS or PMS) were inoculated with 1×10^8 spores. The cultures were incubated for 48 hours at 29°C in the dark with shaking at 150 rpm. The mycelia were filtered through miracloth (Calbiochem, La Jolla CA), washed with cold, sterile water, frozen with liquid nitrogen and stored at -80°C. Frozen mycelia were ground using mortar and pestle with liquid nitrogen and transferred to a tared 125 ml flask. 5 ml of lysis buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT and 1 mM PMSF) per gram of ground mycelia was added to the flask. In addition, 1 ml of protease inhibitor cocktail (Sigma, St. Louis MO - product # P8215) was added to the flask per gram of mycelia. After stirring on ice for 15 minutes, saturated ammonium sulfate was slowly added to a final concentration of 10%. The suspension was then stirred on ice for 15 minutes and then set idle for 15 minutes on ice. Cell debris was then pelleted at 100,000 x g (30 minute spin at 4°C). The volume of the supernatant was then determined using a graduated cylinder and transferred to a 50 ml flask. Then solid ammonium sulfate was added slowly over 1.5 hours to raise the concentration from 10% to 70%. The ammonium sulfate addition was conducted while stirring on ice. After all ammonium sulfate was added, the flask was incubated for 30 minutes on ice without stirring. The protein was pelleted at 10,000 X g (20 minutes at 4°C). The pellet was resuspended in dialysis buffer (15% glycerol, 15 mM Hepes-KOH [pH 7.9], 100 mM KCl, 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail [1 ml per 20 grams of mycelia]) and dialyzed twice in 2 liters dialysis buffer using a 10K slide-a-lyzer (Pierce, Rockford IL). Protein concentration was determined using the BioRad (BioRad, Hercules CA) protein dye reagent following manufacturers instructions. The dialyzed solution was then aliquoted and stored at -80°C.

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nor-1 and *ver-1* Promoter Fragments

The 5' boundary of the *ver-1* promoter region was delineated by the poly A site of the gene immediately upstream, *norA*. The 3' boundary was delineated 25 basepairs downstream of the translational start site. Similarly, the 5' boundary of the *nor-1* promoter region was delineated by the poly A site of the gene immediately upstream - ORF3 (Miller et al., 2003a). The 3' boundary was delineated 34 basepairs downstream for the transcriptional start site. The promoter regions were divided into three subfragments designated R (right), M (middle) and L (left). Overlapping PCR primers were designed for each fragment. The primers for each *ver-1* subfragment carried an additional 8 bases which included a *Bam*HI site. The PCR fragments were cut with *Bam*HI, gel purified (Qiagen, Santa Clarita CA), and labeled using [³²P]-dCTP (NEN, Boston MA) for a fill-in reaction (Ausebel et al., 2003). The *nor-1* promoter PCR subfragments were gel purified (Qiagen, Santa Clarita CA) and end-labeled with [³²P]-ATP (NEN, Boston MA) using Ready-to-Go Kinase (Pharmacia, Piscataway NJ). Both *nor-R* and *ver-R* contain the TATA box, transcriptional and translational start sites as well as the putative AfIR binding site TCGnnnnCGA (Fernandes et al., 1998).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed essentially as described in Current Protocols in Molecular Biology (Ausebel et al., 2003). Five percent acrylamide (80:1 acrylamide:bis-acrylamide) non-denaturing gels were used. 20 fmol of *nor-1* and *ver-1* promoter probes were incubated for 15 minutes at 30°C with 2 µg dIdC, 7.5 µg BSA and competitor (if desired) with 32 mg protein extract (added last). The entire binding reaction volume was

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25 μ l which consisted of 20 μ l of dialysis buffer in order to keep the glycerol concentration of the binding reaction greater than 10%.

ELISA

Enzyme-Linked Immunosorbent Assays were conducted by the method of Pestka (Pestka et al., 1980) using polyclonal antibodies provided by Neogen Corporation (Neogen, Lansing MI). Aflatoxin concentrations are reported as the mean of the triplicate flasks, analyzed twice with the standard deviation represented by errors bars.

RESULTS

Generation of anti-Nor-1 antibody

An *A. parasiticus nor-1* knock-out strain accumulated norsolorinic acid suggesting that Nor-1 catalyzes the conversion of norsolorinic acid to averantin (Chang et al., 1992). Subsequently, recombinant Nor-1 was shown to be able to convert norsolorinic acid to averantin in the presence of NADPH (Zhou and Linz, 1999). Purified recombinant Nor-1:Maltose Binding Protein (Zhou and Linz, 1999) was used as antigen for antibody production. The IgG fraction was purified and used for Western blot analysis (Figure 2.1). In YES shake cultures, *A. parasiticus* SU-1 (wild type) had two major bands at 31 and 28 kDa at both 48 and 60 hours (lanes 2 and 3, respectively). Meanwhile, the Nor-1 disrupted strain *A. parasiticus* Δ Nor-1 did not have either band at 60 hours (lane 1). An *A. parasiticus* SU-1 extract that had been purified using an anti-Nor-1 affinity column resulted in a single 31 kDa band. The predicted size of Nor-1 is also 31 kDa. While the identity of the 28 kDa protein is unknown, these observations

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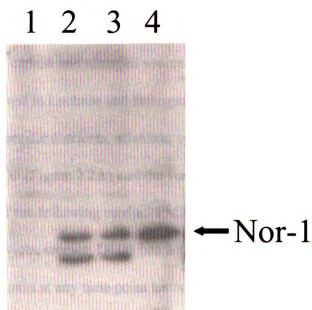


Figure 2.1. Western blot analysis of the native Nor-1 protein from *A. parasiticus* SU-1. Each lane contained 10 mg protein. The primary antibody was the IgG fraction of the antiserum raised against the Nor-1c/MBP fusion protein (10 mg/ml). Lanes: 1, total crude extract (10,000 X g supernatant) from the *nor-1* disrupted strain *A. parasiticus* Δ Nor-1 cultured in YES liquid medium for 60 h; 2 and 3, total crude extract from the wild type *A. parasiticus* SU-1 cultured in YES liquid medium for 48 h and 60 h respectively; 4, the native Nor-1 protein (31 kDa) purified with anti-Nor-1c/MBP fusion protein PAb affinity column from the total crude extract of *A. parasiticus* SU-1 cultured in YES medium for 60 h.

suggest that the 31 kDa protein is the native Nor-1 protein encoded by the *nor-1* gene. In addition, the Nor-1 antibody is specific for Western blot analysis for native Nor-1 protein.

Carbon, nitrogen and zinc affect aflatoxin production and aflatoxin gene expression

The effects of zinc, carbon and nitrogen source on aflatoxin synthesis have been previously reported (reviewed in Luchese and Harrigan, 1993). To verify previously observed effects of these specific nutrients, aflatoxin synthesis was measured in the *nor-1::GUS* reporter strain D8D3 (Figure 2.2A) and the *ver-1::GUS* reporter strain I4 (Figure 2.2B) separately, in each of the following media: PMS (D8D3 only), NMS, GMS₀, and GMS. Consistent with previous studies, D8D3 batch cultures in PMS did not produce detectable amounts of aflatoxin at any time point tested. Aflatoxin levels in NMS batch cultures were either non-detectable (D8D3 all three time points and I4 at 48 hours) or detectable but at greatly reduced concentrations compared to GMS shake cultures. In GMS₀ batch cultures of D8D3 and I4, a low constant level of aflatoxin was present at all three time points tested.

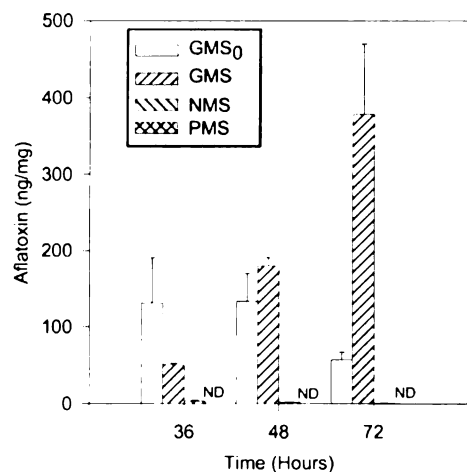
To examine the nutrient effects on accumulation of aflatoxin biosynthesis transcripts and proteins in D8D3 and I4, Northern and Western analysis were performed. Consistent with the data on aflatoxin synthesis, native Nor-1 protein and *nor-1* transcript accumulated in GMS cultures at all three time points for both D8D3 (Figure 2.3A,B) and I4 (data not shown). Native Nor-1 protein and *nor-1* transcript accumulation in PMS cultures was undetectable for D8D3 (Figure 2.3A,B). With D8D3 and I4, NMS cultures did not contain detectable amounts of *nor-1* transcript or Nor-1 protein except for a light signal for *nor-1* transcript at 72 hours with D8D3 in NMS (data not shown). Native

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A



B

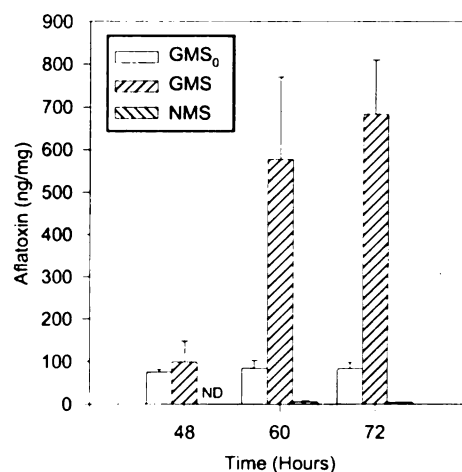
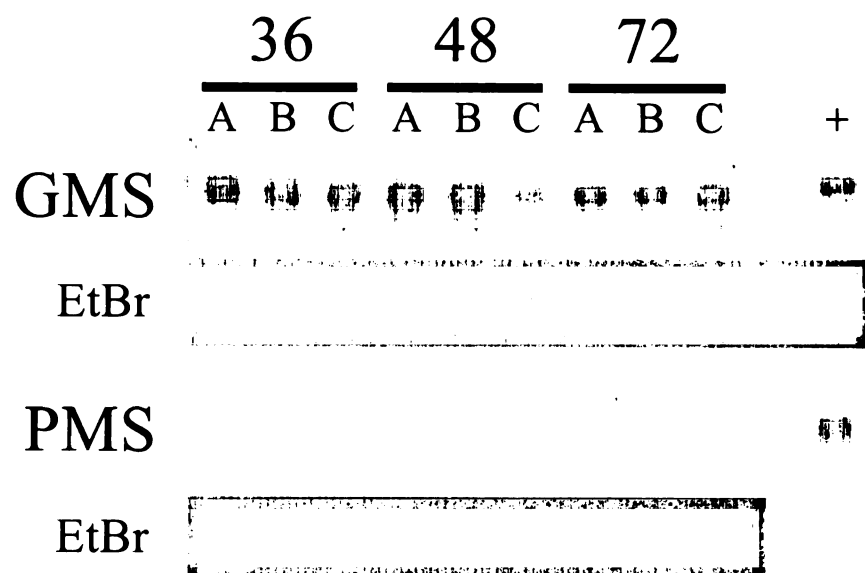


Figure 2.2. Aflatoxin production by the reporter strains *A. parasiticus* D8D3 (A) and I4 (B) grown in GMS₀, GMS, NMS and PMS (D8D3 only) to 36, 48 and 72 hours (D8D3) or 48, 60 and 72 hours (I4). Aflatoxin concentration of the growth media was determined by competitive-direct ELISA using polyclonal antibodies. Aflatoxin concentrations are reported as the average of the triplicate flasks, analyzed twice, with the standard deviation represented by errors bars. Non-detectable activity is represented by ND.

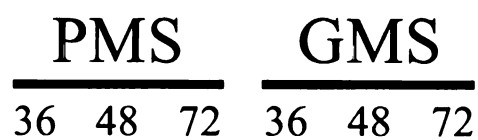
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Figure 2.3. *nor-1* transcript and Nor-1 protein accumulation and *aflR* transcript accumulation by the reporter strain *A. parasiticus* D8D3. (A) *nor-1* transcript accumulation assessed by Northern hybridization analysis. For the time points (36, 48 and 72 h), each replicate flask is presented (A, B and C). Top panel is RNA from GMS cultures and bottom panel is RNA from PMS cultures. The lane designated “+” has RNA from a 48 h *A. parasiticus* SU1 culture in YES medium. Equal loading of RNA is demonstrated by ethidium bromide staining of RNA as shown in the panels marked “EtBr”. (B) Nor-1 protein accumulation by Western blot analysis. One flask per time point (36, 48 and 72 h) was analyzed from PMS and GMS cultures. (C) *aflR* transcript accumulation assessed by Northern hybridization analysis. For the time points (36, 48 and 72 h), each replicate flask is presented (A, B and C). Top panel is RNA from GMS cultures and bottom panel is RNA from PMS cultures. The lane designated “+” has RNA from a 48 h *A. parasiticus* SU1 culture in YES medium. Equal loading of RNA can be determined from part A in the panels marked “EtBr”.

A



B



C

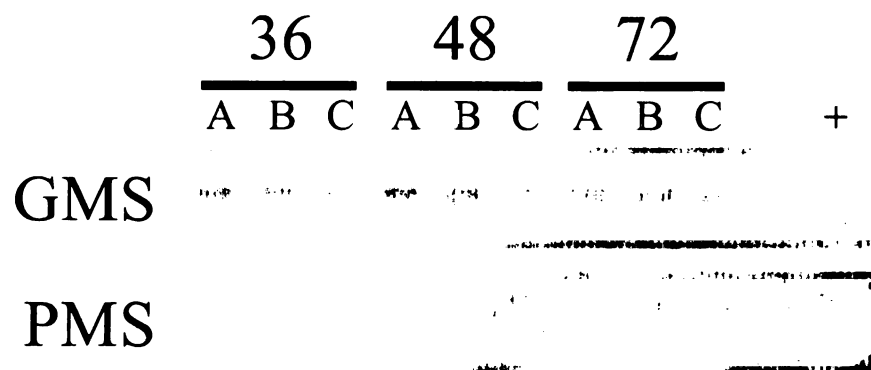


Figure 2.3

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Nor-1 protein and *nor-1* transcript accumulated at the 36 hour time point for GMS₀ but were non-detectable at 48 and 72 hours with D8D3 and I4 (data not shown). Analysis of total protein from GMS₀ on SDS-PAGE revealed that proteins at 48 and 72 hours displayed extensive proteolysis which was much less severe at 24 hours (data not shown). Native Ver-1 protein and *ver-1* transcript levels in I4 followed a very similar pattern as native Nor-1 protein and *nor-1* transcript accumulation in D8D3 (data not shown).

AflR is a transcription factor that is required for aflatoxin biosynthesis gene transcription. To examine the nutrient effects on accumulation of *aflR* transcripts in D8D3 and I4, Northern hybridization analyses were performed (Figure 2.3C). *aflR* transcript accumulated to greater levels at all three time points for D8D3 and I4 (data not shown) in GMS cultures compared to PMS cultures (Figure 2.3C). *aflR* transcript accumulation in NMS and GMS₀ in D8D3 and I4 was non-detectable except for the first time point with GMS₀. Transcript steady states of the aflatoxin transcription factor *aflR* coincides *nor-1* and *ver-1* transcript and protein accumulation.

Nutrient effects are in part regulated at level of transcription

The previous results suggested that rates of transcript accumulation in part mediate changes in aflatoxin synthesis, but do not clarify if accumulation occurs via changes in transcription rate or transcript stability. Reporter assays with D8D3 (data not shown) and I4 (Figure 2.4) were used to analyze if zinc concentration, carbon source and nitrogen source affect transcriptional regulation of the aflatoxin genes *nor-1* and *ver-1*. GUS activity of I4 GMS cultures was 9 fold higher than I4 GMS₀ cultures at 72 h (Figure 2.4). I4 GMS cultures had 60 fold higher GUS activity than I4 NMS cultures (Figure 2.4). Results with D8D3 activities with GMS, GMS₀ and NMS were similar to I4 (data

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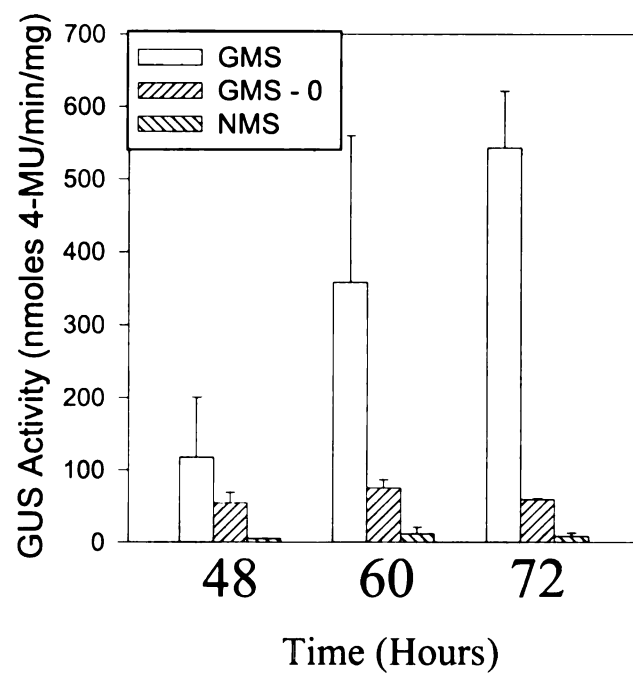


Figure 2.4. β -glucuronidase (GUS) expression by the reporter strains *A. parasiticus* I4.

Cultures were grown in GMS, GMS₀ (GMS - 0) and NMS for 48, 60 and 72 h.

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not shown). In addition, the GUS activity of D8D3 PMS cultures was non-detectable (data not shown). While we cannot eliminate transcript stability as a factor, transcriptional activation of *nor-1* and *ver-1* is at least partially responsible for the differences in aflatoxin production under the conditions tested.

Nutrients affect DNA promoter binding by cellular proteins

In order to determine if DNA binding proteins mediate *nor-1* promoter activity, electrophoretic mobility shift assays (EMSA) were performed with *A. parasiticus* SU1 and AFS10 protein extracts. The DNA probes were generated from the promoter region of *nor-1* (Figure 2.5). To test for specificity, unlabeled oligonucleotides were used as competitors (250 fold molar excess) for protein binding. The promoter fragment nor-R, which contains an AflR binding site, produced two shifted complexes with both *A. parasiticus* SU1 (wild type) and *A. parasiticus* AFS10 (AflR knock-out) extracts from PMS cultures. Of these two shifted complexes, the faster migrating complex appears to be specific. The promoter fragment nor-R* is the same as nor-R except the AflR binding site was altered (TCGgccagCGA to AGTttaaCAG). Since nor-R and nor-R* are both effective competitors for the specific complex, the AflR *cis*-acting site is not responsible for the shifted complex. In addition, since the specific shifted complex appears in AFS10 extracts, the protein responsible for the shifted complex appears to be AflR independent.

The nor-R oligonucleotide produced three shifted complexes with AFS10 extracts from GMS medium (Figure 2.6). nor-R and nor-R* did not compete for any of these three shifted complexes. Consequently, all three complexes are non-specific. The nor-R oligonucleotide produced two shifted complexes with SU1 extracts from GMS medium. Of the two shifted complexes, the intensity of the faster migrating complex was reduced

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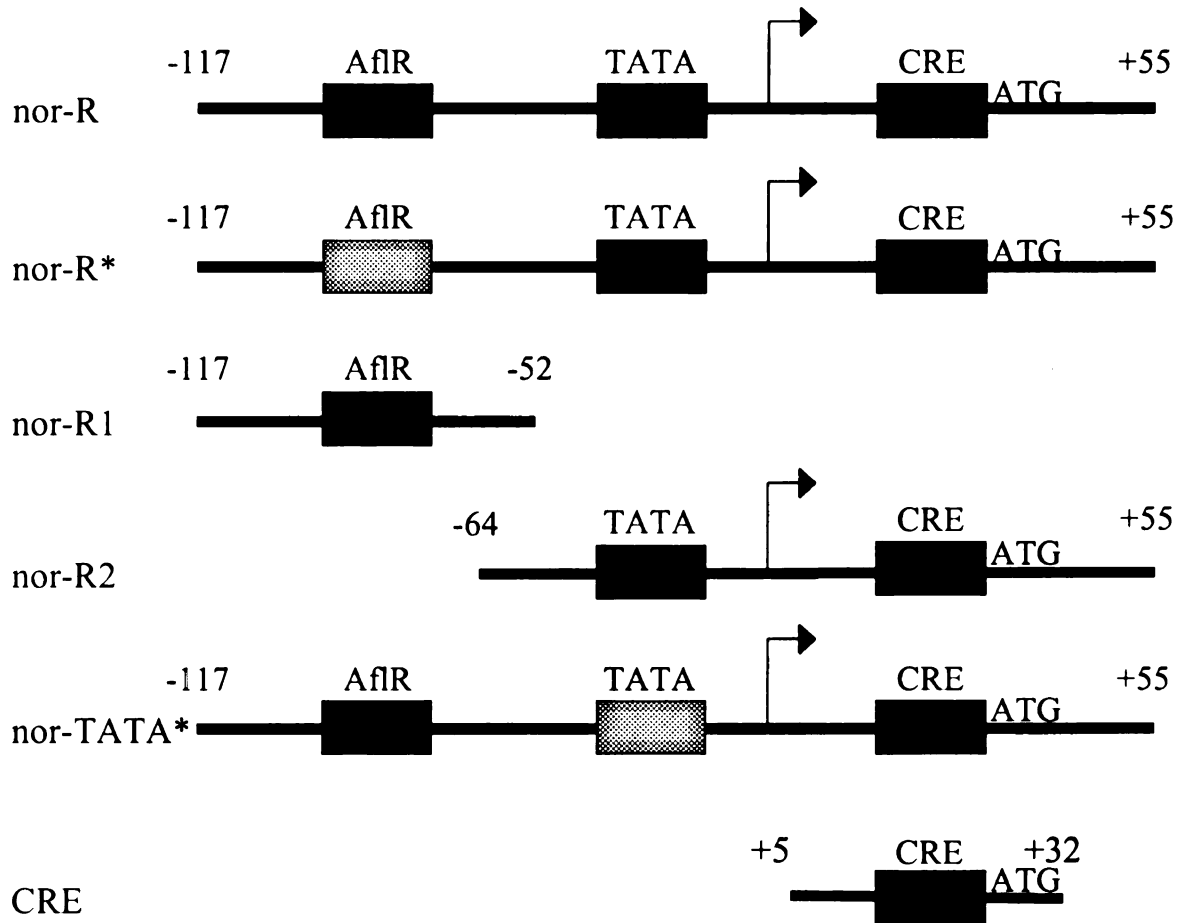


Figure 2.5. Map of EMSA oligonucleotides used for electrophoretic mobility shift assay (EMSA). Three putative *cis*-acting sites are boxed, AfIR TATA and CRE. For nor-R*, the AfIR site has been mutated (TCGgccagCGA to AGTttaaCAG). For nor-TATA*, the TATA box was mutated (5'-ATATATAG-3' to 5'-GTTTAAAC-3').

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strain	-	S	S	S	A	A	A	S	S	S	A	A	A
medium	-	P	P	P	P	P	P	G	G	G	G	G	G
competitor	-	-	R	R*	-	R	R*	-	R	R*	-	R	R*

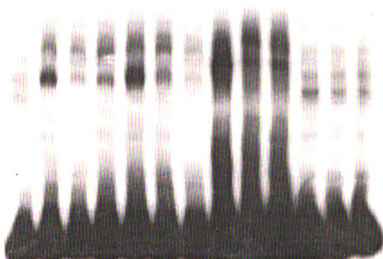


Figure 2.6. EMSA of nor-R. Protein extracts were collected from two different *A. parasiticus* strains, SU1 (S) and AFS10 (A). Each strain was grown in two different media, PMS (P) and GMS (G). 32 mg of protein was used per lane. The two competitors (250 fold excess) used were nor-R (R) and nor-R* (R*).

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in the presence of both nor-R and nor-R* as competitors. Since nor-R and nor-R* are both effective competitors for the specific complex, the AflR *cis*-acting site is not responsible for the shifted complex. The protein responsible for the specific shifted complex with SU1 GMS extracts is dependent on functional AflR for activity because the AFS10 (AflR knock-out strain) extract lacked this shifted complex. In addition, the mobility of the specific shifted complex appears different than the specific shifted complex seen with the PMS extracts (both SU1 and AFS10) suggesting that different proteins may be responsible for the specific complexes.

In order to localize the specific DNA binding site in the SU1 GMS extracts, additional competition experiments were performed (Figure 2.7) with various oligonucleotide competitors (Figure 2.5). For example, nor-R was divided into two overlapping oligonucleotides, nor-R1 and nor-R2. Since nor-R2 was an effective competitor while nor-R1 was not, additional competitors were designed to test potential sites in nor-R2. nor-TATA* is the same as nor-R except the TATA box has been substituted (5'-ATATATAG-3' to 5'-GTTTAAAC-3'). nor-TATA* is an effective competitor suggesting that the TATA box is not responsible for the shifted complex. Lastly, an oligonucleotide that overlaps the translational start point, CRE, was an effective competitor. The specific complex seen with *A. parasiticus* SU1 protein from GMS cultures bound to the CRE1 region. The CRE1 binding protein is likely a nor-1 transcriptional activator.

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strain	-	S	S	S	S	S	S
medium	-	G	G	G	G	G	G
competitor	-	-	R	RT*	R1	R2	CRE

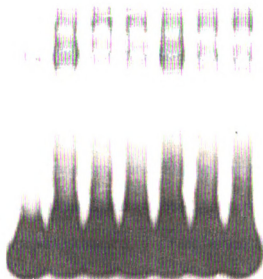


Figure 2.7. nor-R competition EMSA. Protein extracts were collected from *A. parasiticus* SU1 (S) grown in GMS (G). 32 mg of protein was used per lane. The competitors used were: nor-R (R), nor-TATA* (RT*), nor-R1 (R1), nor-R2 (R2) and CRE.

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DISCUSSION

The effects of environmental and nutritional factors such as temperature, pH, carbon source, nitrogen source and zinc concentration on aflatoxin production have been previously studied (reviewed in Luchese and Harrigan, 1993). However, nutritional factors such as carbon source, nitrogen source and zinc concentration provide useful tools in studying the molecular mechanisms of aflatoxin biosynthesis gene regulation. In addition, the creation of a *nor-1::GUS* (D8D3) strain, a *ver-1::GUS* strain (I4) and an *aflR* knock-out strain (AFS10) permitted the study of these nutritional factors and aflatoxin gene transcriptional regulation. We presented a comprehensive analysis of the use of nutritional components as tools to study aflatoxin gene transcriptional regulation.

NMS and PMS cultures produced low or non-detectable levels of *nor-1* and *ver-1* transcript, Nor-1 and Ver-1 protein and aflatoxin. However, GMS cultures produced significant levels of *nor-1* and *ver-1* transcript, Nor-1 and Ver-1 protein and aflatoxin. GUS reporter activities confirmed that the increase in *nor-1* and *ver-1* transcript steady states in GMS was due, at least in part, to transcriptional activation. However, we can not rule out transcript stability because the *nor-1::GUS* and *ver-1::GUS* transcripts may also be subject to the same transcript stability process. *aflR* transcript steady state was also higher in GMS cultures. Insertion of an additional copy of *aflR* results in the upregulation of aflatoxin biosynthesis (Chang et al., 1995). In addition, *aflR* transcript and AfIR protein accumulation in various nutritional and environmental conditions have been shown to be consistent with aflatoxin production, in that culture conditions with higher *aflR* steady states also have higher concentrations of aflatoxin (Liu et al., 1999). GMS (glucose, ammonium and 10mM zinc) induces aflatoxin gene transcription,

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possibly via the upregulation of aflR.

GMS₀ cultures appear similar to GMS cultures at the earliest time point. However, at the later time points, the amount of aflatoxin in the culture medium, *nor-1* and *ver-1* transcript and protein steady states and *nor-1* and *ver-1* transcriptional activation either stayed the same or decreased. Analysis of the protein extracts from the GMS₀ cultures on SDS-PAGE gels revealed that the protein extract was subject to extensive proteolysis. Repeated attempts to extract GMS₀ cultures revealed the same proteolysis. The omission of zinc from the culture medium not only results in a decrease in aflatoxin production but also in growth (Bennett et al., 1979). Consequently, we hypothesize that once the growing culture runs out of zinc, the fungal cells lyse and the proteins degrade. If true, the lack of transcriptional activation in GMS₀ cultures is not due to a specific repression of aflatoxin biosynthesis.

Since GMS cultures induce *nor-1* transcriptional activation, protein extracts from GMS cultures are a logical place to look for transcriptional activators specific for aflatoxin. Using a 160 base pair *nor-1* promoter fragment as probe (nor-R), electrophoretic mobility shift assays (EMSA) with *A. parasiticus* SU1 (wild type) GMS extracts produced a single specific shifted complex. Using various competitors for the specific nor-R shifted complex allowed us to localize the protein binding site to a region that we designated CRE1. The protein that binds to CRE1 appears to be dependent on AflR because the *A. parasiticus* AFS10 (*aflR* knock-out) lacks this shifted complex. A limitation with EMSA is that it is an *in vitro* assay. However, preliminary experiments indicate that the CRE1 site has functional significance *in vivo* as well (Dr. Ludmila Roze, personal communication). We did not detect AflR binding to nor-R consistent with previous studies with recombinant AflR (Ehrlich et al., 1999b). However, this AflR

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binding site was shown to be necessary for *nor-1* transcriptional activation (Miller et al., 2003a). It is unknown why our *A. parasiticus* SU1 (wild type) protein extract lacks AfIR activity.

AfIR is necessary for *nor-1* transcriptional activation (Miller et al., 2003a). In addition, forced expression of AfIR using an inducible vector results in aflatoxin production even in non-aflatoxin inducing conditions (Flaherty and Payne, 1997). Nutrients such as nitrogen source may affect aflatoxin production by modulating *aflR* expression. AreA, a global nitrogen regulatory protein, has been shown to bind to the *aflR* promoter (Chang et al., 2000). The number of AreA binding sites in the *aflR/aflJ* intergenic region may reflect differences between different strains and the amount of aflatoxin they make in response to different nitrogen sources (Ehrlich and Cotty, 2002). Additional experiments are needed to elucidate the mechanisms for transcriptional activation of aflatoxin biosynthetic genes in response to nitrogen and carbon sources.

ACKNOWLEDGMENTS

Dr. Renqing Zhou generated the anti-Nor-1 polyclonal antibody and provided Figure 2.1. All other experiments described in Chapter 2 were performed by Matt Rarick and Michael Miller. Chapter 2 will be submitted for publication in the near future.

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CHAPTER 3

Chromosomal Location Plays a Role in Regulation of Aflatoxin Gene Expression in

Aspergillus parasiticus

INTRODUCTION

Aflatoxins are highly toxic secondary metabolites produced predominantly by the fungi *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Council for Agricultural Science and Technology, 2003). These toxins frequently contaminate food and feed crops, including peanuts, corn, cottonseed, and tree nuts, and generate a large health and economic impact in the United States and in other countries (reviewed in Council for Agricultural Science and Technology, 2003). To help eliminate aflatoxin contamination of food and feed, we have studied the mechanisms that regulate aflatoxin gene expression. This knowledge will be used to design novel, effective control strategies to reduce aflatoxin contamination of crops in the field and during storage.

Aflatoxin biosynthesis is a complex process that requires at least 16 different enzymatic steps (Bhatnagar et al., 1994) encoded by up to 25 individual genes (Keller and Adams, 1995). These genes are clustered in *A. flavus*, *A. parasiticus* and *A. nidulans* (*A. nidulans* synthesizes the penultimate pathway intermediate, sterigmatocystin). It was previously hypothesized that clustering of aflatoxin genes may allow coregulation in response to environmental cues, although no conclusive data were reported (Trail et al., 1995b).

Preliminary evidence for a role for clustering in aflatoxin gene regulation was

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reported by Liang et al. (Liang et al., 1997). The promoter of the aflatoxin biosynthesis structural gene *ver-1* was fused to *uidA* (encodes β -glucuronidase [GUS]) (Jefferson, 1989) to generate a reporter plasmid, pHD6.6, containing *niaD* (encodes nitrate reductase) as a selectable marker. pHD6.6 was transformed into *A. parasiticus* NR1 (*niaD*), resulting in integration predominantly at the *ver-1* or *niaD* locus via homologous recombination. Single-copy integration of pHD6.6 at *niaD* resulted in a 500-fold reduction in *ver-1* promoter activity when compared with single-copy integration at the *ver-1* locus; however, the temporal pattern of expression appeared to be similar at both loci. One explanation for reduced *ver-1* promoter function at the *niaD* locus is that the location in the aflatoxin cluster results in positive, position-dependent regulation of *ver-1* expression. An alternative hypothesis is that the expression of *ver-1* integrated at *niaD* is negatively influenced by *niaD* regulation. In the absence of preferred nitrogen sources and in the presence of nitrate, *niaD* is expressed. Under the rich growth conditions tested by Liang et al. (Liang et al., 1997), *niaD* may have been repressed, and therefore the lack of *ver-1* expression at the *niaD* site could have resulted from *niaD*-dependent regulation.

Subsequently, the promoter of the aflatoxin biosynthesis gene *nor-1* was fused to GUS to generate a reporter plasmid, pAPGUSNN-B, containing *niaD* as a selectable marker (Chiou et al., 2002). Transformants with pAPGUSNN-B integrated at the *niaD* locus had no detectable GUS activity while *nor-1* integrants had GUS activity (Chiou et al., 2002). In addition, GUS transcript and protein accumulation correlated with *nor-1* transcript and protein accumulation in terms of timing and amount (Chiou et al., 2002). Because *niaD* dependent regulation could account for the absence of expression at *niaD* for both *nor-1*::GUS (Chiou et al., 2002) and *ver-1*::GUS (Liang et al., 1997), a third chromosomal location was analyzed using pAPGUSNP, which contained *nor-1*::GUS

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plus *pyrG* (encodes OMP decarboxylase) as a selectable marker. GUS expression was detectable only when pAPGUSNP integrated at *nor-1* and was not detectable at *pyrG*, even under growth conditions that required *pyrG* expression.

Our hypothesis was that the aflatoxin biosynthesis genes are subject to gene-cluster-dependent regulation. To test our hypothesis, we wanted (i) to generate a new *nor-1*::GUS reporter construct (pNANG-3) that enables easy promoter replacement and (ii) to validate the positional-dependent regulation by correlating the site of integration with solid culture GUS activity for pBNG3.0 transformants. We generated a series of plasmids that enable a *nor-1* promoter to be easily amplified by PCR and ligated into a *nor-1*::GUS reporter plasmid containing *niaD* as a selectable marker (pNANG-3). Using a solid culture GUS assay, we screened pBNG3.0 (3.0 kb *nor-1* promoter fragment ligated into pNANG-3) transformants for GUS activity. GUS+ transformants were tested by a rapid PCR site of integration assay which identified all 21 GUS+ transformants as either 5' *nor-1* (12) or 3' *nor-1* (9) integrants. Conversely, all 22 GUS- transformants were negative for 5' *nor-1* and 3' *nor-1* integration. Southern hybridization analysis was used to confirm 3' *nor-1* integration of all 8 preliminary 3' *nor-1* integrants tested. These results confirm the utility of the *nor-1*::GUS reporter plasmid for analyzing *nor-1* promoter function and the positional-dependent regulation previously reported (Liang et al., 1997 and Chiou et al., 2002).

MATERIALS AND METHODS

Strains and growth conditions

Escherichia coli DH5 α F'c [F' *endA1 hsdR17* ($r_k^- m_k^-$) *supE44 thi-1 recA gyrA*

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(NaI^r) $\Delta relA1$ (*lacZYA argF*)_{u169}:(m80 $\Delta lacZ$ M15)] (Invitrogen, Carlsbad, CA) was used to amplify plasmid DNA using standard procedures (Ausubel et al., 2003). *Aspergillus parasiticus* NR1 (*niaD*) was used as the recipient strain for all fungal transformations (Horng et al., 1990).

To measure solid culture GUS activity, YES agar (2% yeast extract, 6% sucrose; pH=5.8) plates were used.

Plasmid Constructs

(i) pNebN1 (Figure 3.1B). pNEB193 (Figure 3.1A - New England Biolabs, Beverly, MA) was digested with *Bam*HI and the protruding ends were filled in by Klenow fragment. A *Not*I linker (5'-AGCGGCCGCT-3') was then ligated onto the blunt ends. The DNA was then digested with *Not*I, agarose gel purified using QIEX II (Qiagen, Valencia, CA) and then re-ligated. Bacterial transformants were screened by digesting mini-prep DNA with *Not*I. The resulting plasmid (pNebN1) no longer has a *Bam*HI site and can no longer be used for blue/white screening.

(ii) pNiaD-A1 (Figure 3.1C). A 7.4 kb *Xho*I/*Sal*I fragment from pSL82 (Horng et al., 1990) was agarose gel purified using QIEX II (Qiagen, Valencia, CA) and then ligated into the *Sal*I site of pNebN1. Bacterial transformants were screened using the colony hybridization technique (Ausubel et al., 2003). The *Xho*I/*Sal*I 7.4 kb fragment was radioactively labeled using the random primed labeling kit (Roche Applied Science, Indianapolis, IN) and used as probe. Only 6 of 600 clones were positive in the colony hybridization. The positive clones were further analyzed by digesting plasmid DNA with *Pst*I. Plasmids in the “A” orientation result in 4.2 and 5.9 kb DNA fragments while plasmids in the “B” orientation result in 3.2 and 6.9 kb fragments. Functionality of the

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Figure 3.1. Restriction endonuclease maps of relevant plasmids. (A) pNEB193 (New England Biolabs, Beverly, MA). This plasmid is a modification of pUC19 that incorporates additional restriction endonuclease sites in the multiple cloning sites. These additional restriction enzymes recognize 8 bp sequences and consequently are less likely to exist in the DNA being cloned. (B) pNebN1. This plasmid has a *NotI* site that replaces a *BamHI* site in pNEB193 by insertion of a *NotI* linker. (C) pNiaD-A1. A 7.4 kb *XhoI/SalI* fragment from pSL82 (Hornig et al., 1990) that contains the *niaD* selectable marker was cloned into the *SalI* site of pNebN1. (D) pNANG-3. In addition to carrying the *niaD* selectable marker, pNANG-3 carries a small part of the *nor-1* coding sequence (10 amino acids) fused to the β -glucuronidase (*uidA* or GUS) coding sequence which is in turn fused to the 2 kb *nor-1* 3' terminator fragment. The small numbers of codons that were changed in the *nor-1* coding sequence were all acceptable based on codon usage and maintained the correct reading frame. The 4 kb GUS/*nor-1* terminator fragment was amplified by PCR using pAPGUSNN-B (Figure 3.1F) as template with primers that had *NotI* (5') and *AscI* (3') tails. Appropriate promoter pieces, amplified by PCR using primers with *NotI* (and *PacI* if directionally cloned) can be cloned into pNANG-3. (E) pBNG3.0-3F. This plasmid contains a 3 kb PCR-amplified *nor-1* promoter piece cloned into the *NotI* site of pNANG-3. (F) pAPGUSNN-B. Original *nor-1*::GUS reporter plasmid constructed by Wilson (Chiou et al., 2002).

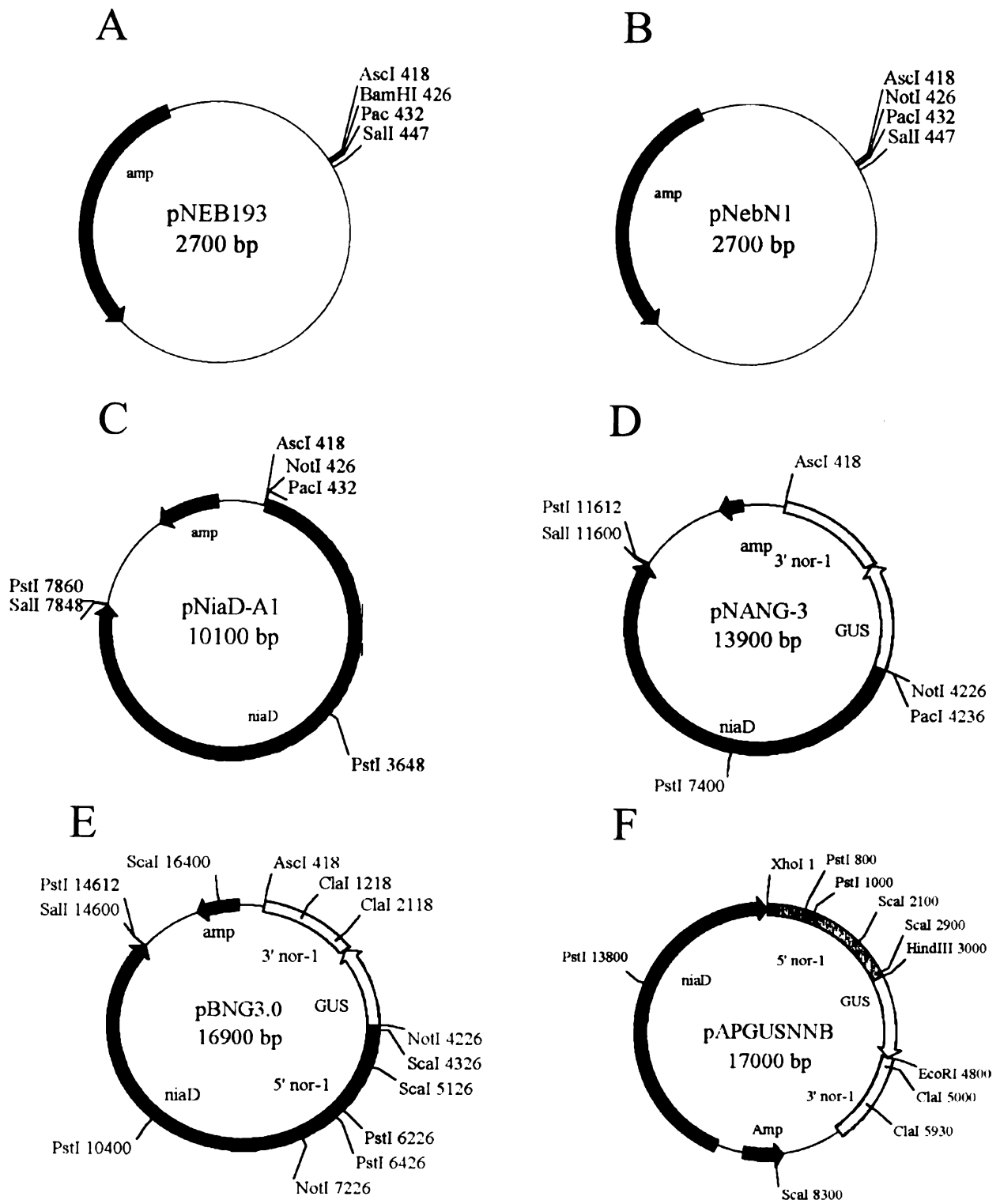


Figure 3.1

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niaD gene was tested by performing a fungal transformation with pNiaD-A1, pNiaD-B2 and the positive control pSL82 (Horng et al., 1990). The transformation frequency for pNiaD-A1, pNiaD-B2 and pSL82 was 17 colonies/ μ g, 19.2 colonies/ μ g and 17.6 colonies/ μ g respectively.

(iii.) pNANG-3 (Figure 3.1D). To insert the *uidA* gene (GUS), the reporter construct pAPGUSNN-B (Chiou et al., 2002) was utilized as a template for PCR. Primers were designed to amplify a 4 kb fragment that contained the *nor-1* terminator region, GUS coding sequence and the *nor-1* coding sequence upstream of the fusion point. The primers contained restriction sites (underlined residues) to facilitate cloning: the 5' primer (5'-TAGCGGCCGGATCAAGAGAAGCTCTATACG-3') contained *NotI* and the 3' primer (5'-TTGCGCGCCCCTCGATGATGCTCTG-3') contained *AscI*. Since the *NotI* site was located within the coding sequence, care was taken to maintain the correct reading frame. The 4 kb *NotI/AscI* PCR product was ligated into the *NotI* and *AscI* sites of pNiaD-A1. Bacterial transformants were screened by colony hybridization with the *NotI/AscI* PCR product as probe (same procedure as with pNiaD-A1). More than 90% of transformants were shown to carry the insert. Selected positives were then screened by restriction enzyme analysis with *PstI* (expected sizes: 4.2 and 9.7 kb). Because PCR mediated mutations in the *NotI/AscI* fragment may have occurred, four different positive clones (pNANG-1, -2, -3 and -4) were saved.

(iv.) pBNG3.0-3F (Figure 3.1E). A 3 kb *nor-1* promoter piece was amplified by PCR with pAPGUSNN-B as template and appropriate primers with *NotI* tails (5'-TCGCGGCCGCCTAAGTGATCCATTATTATGTC-3' and 5'-TTGCGGCCGCCTCCTTGTCTCTGTAAGATAAAA-3'). In this way, the *nor-1* promoter could be easily removed and replaced with other putative promoters to test for function. Both pNANG

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(pNANG-1, -2, -3 and -4) and the PCR fragment were digested with *NotI* and then gel purified using QIEXII (Qiagen, Carlsbad, CA). The promoter insert was then ligated into all four vectors. Bacterial transformants were screened by colony hybridization (see pNiaD-A1) with the *nor-1* promoter PCR fragment used as probe. Orientation of the promoter insert was determined by digesting plasmid DNA with *PstI* with the proper orientation resulting in 8.6, 4.2, 4.0 and 0.2 kb fragments. The nucleotide sequence from pBNG3.0-1, -2, -3 and -4 was analyzed from the *nor-1*::GUS fusion site to 322 bp upstream from the *nor-1* transcriptional start site and confirmed the correct nucleotide sequence in this reporter construct. pBNG3.0-3F was used for fungal transformations.

(v.) pAPGUSNN-B (Figure 3.1F). The pAPGUSNN-B (Chiou et al., 2002) *nor-1*::GUS fusion construct was derived from pAPGUSN (Chiou et al., 2002) by insertion of a 7.4 kb *Sall/XhoI* fragment carrying *niaD* from pSL82 (Horng et al., 1990). The *nor-1* promoter region consists of a 3 kb *HindIII* DNA restriction fragment carrying flanking sequences, promoter, and the first 21 amino acid residues of Nor-1 fused in frame to *uidA* from *E. coli* (Jefferson 1989). The *nor-1* terminator is a 1.8 kb *EcoRI/Sall* genomic DNA fragment containing the last six amino acids of Nor-1, the transcription terminator and flanking sequences.

Transformation and Identification of the site of plasmid integration

(i) Transformation. Transformation of *A. parasiticus* protoplasts was performed as described by Horng et al. (1990). 10 µg of pBNG3.0-3F was transformed into 2.7×10^7 NR-1 (*niaD*) protoplasts. Selection of *niaD*⁺ colonies resulted in 276 total transformants (transformation frequency = 1.0×10^{-6} CFU/protoplast/µg plasmid).

(ii) Rapid DNA extraction procedure. *A. parasiticus* transformants were

inoculated onto Czapek-Dox (Difco, Detroit, MI) agar plates and incubated for 3-4 days in the dark at 29°C. A sterile pipette tip was used to scrape some mycelia from the surface of the agar which was deposited in a 1.5 mL screw cap tube containing 100 µL TE (10 mM Tris-Cl, 1 mM EDTA, pH = 8). The sample was then boiled for 5 minutes and centrifuged for 10 minutes at 15,000 X g. The resulting supernatant was used as a template for PCR.

(iii) 3' PCR analysis. The primers for 3' integration were JL 102 (5'-CGCAAGG TGAGGGTTCGAACCGAGG-3') and JL 103 (5'-CCGCAGCAGGGAGGCAAACAAT GAA-3'). Each 50 µL PCR contained: 2.5 µL crude template, 1X reaction buffer for *Pfu* Turbo (Stratagene, La Jolla, CA), 200 µM dNTP, 1 mM MgCl₂, 50 pmol JL 102, 50 pmol JL103, 2.5 units *Pfu* Turbo (Stratagene, La Jolla, CA) and water. Reaction conditions included an initial denaturation step at 95°C for 3 minutes followed by 35 cycles; each cycle consisted of 95°C for 1 minute, 68°C for 1 minute and 72°C for 3 minutes. The reaction was terminated by incubation at 72°C for 10 minutes. Following PCR, 20 µL of reaction mixture was loaded on a 1% agarose gel. After electrophoresis, the occurrence of a 2.1 kb DNA fragment demonstrated a positive reaction for 3' integration.

(iv) 5' PCR analysis. The primers for 5' integration were JL99 (5'-TTTCACGGG TTGGGGTTTCTACAGG-3') and JL100 (5'-GACGGGCAACCTCTTTACAAACATC-3'). Each 50 µL PCR contained: 2.5 µL crude template, 1X reaction buffer, 200 µM dNTP, 1 mM MgCl₂, 50 pmol JL 99, 50 pmol JL100, 2.5 units *Pfu* Turbo (Stratagene, La Jolla, CA) and water. Reaction conditions included an initial denaturation step at 95°C for 3 minutes followed by 35 cycles; each cycle consisted of 95°C for 1 minute, 62°C for 1 minute and 72°C for 5 minutes. The reaction was terminated by incubation at 72°C for

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10 minutes. Following PCR, 20 μ L of reaction mixture was loaded on a 1% agarose gel. A 3.1 kb DNA fragment demonstrated a positive reaction for 5' integration.

(v) Southern hybridization analysis. Genomic DNA was purified from *A. parasiticus* cultures shaken for 48 h in 100 ml YES (2% yeast extract, 6% sucrose and pH=5.8) liquid medium at 29°C with five, 6 mm glass beads (Skory et al., 1993). The DNA was electrophoresed under standard conditions (Ausubel et al., 2003). Southern hybridization analysis was performed according to standard procedures (Ausubel et al., 2003). 2.5 μ g of genomic DNA was digested with *ScaI* and probed with a 900 bp *Clal* fragment isolated from the *nor-1* terminator region of pAPGUSNN-B (Chiou et al., 2002). Digestion of DNA from the recipient strain NR-1 generates a 3.0 kb DNA restriction fragment while a 3' *nor-1* integrant results in 3.7 and 4.0 kb DNA restriction fragments.

Reporter Assays

(i) Solid culture GUS Assay. Autoclaved Nytran SPC (Schleicher & Schuell, Keene, NH) membranes were first placed on top of YES (2% yeast extract, 6% sucrose, 1.5% agar and pH=5.8) agar plates. Transformants were then transferred via sterile toothpick to the Nytran membrane. After incubation for 46 h at 29°C in the dark, the Nytran filters were removed, frozen in liquid nitrogen and thawed at room temperature (2 repetitions) and then incubated for 2 h with GUS substrate solution that includes 0.04% of the colorimetric GUS substrate (X-Glu: 5-bromo-4-chloro-3-indolyl β -D-glucuronide) in GUS reaction buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 0.07% β -mercaptoethanol) and visually evaluated.

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RESULTS

Generation of transformants

Previously, *nor-1::GUS* reporter strains generated using pAPGUSNN-B (Figure 3.1F) demonstrated the usefulness and validity of the GUS reporter system (Chiou et al., 2002). A drawback with pAPGUSNN-B was the inability to replace the 3.0 kb *nor-1* promoter with a different *nor-1* promoter. Consequently, a new *nor-1::GUS* reporter plasmid was designed that utilized unique restriction enzyme sites to facilitate the independent cloning of a *nor-1* promoter. A 3.0 kb *nor-1* promoter fragment was amplified with PCR using primers that had *NotI* tails. The promoter fragment was then cloned into the *NotI* site in pNANG-3 (Figure 3.1D) resulting in pBNG3.0-3F (Figure 3.1E). Using a similar cloning strategy, several different *nor-1* promoter fragments have been fused to GUS and been analyzed (Miller et al., 2003a and Miller et al., 2003b).

Figure 3.2 outlines the procedure used to generate, screen and test pBNG3.0-3F transformants. Ten μg of pBNG3.0-3F was transformed into 2.7×10^7 NR-1 (*niaD*) protoplasts. Selection of *niaD*⁺ colonies resulted in 276 total transformants (transformation frequency = 1.0×10^{-6} CFU/protoplast/ μg plasmid). The transformation frequency was similar to previously reported frequencies with the related pAPGUSNN-B plasmid (Chiou et al., 2002).

Phenotype Screening of Transformants

Colonies grown for 42-48 h on YES agar were used for solid culture GUS analysis. GUS positive fungal colonies convert the GUS substrate X-glu (5-bromo-4-chloro-3-indolyl β -D-glucuronide) to a blue product. After 1-2 h, the blue color is

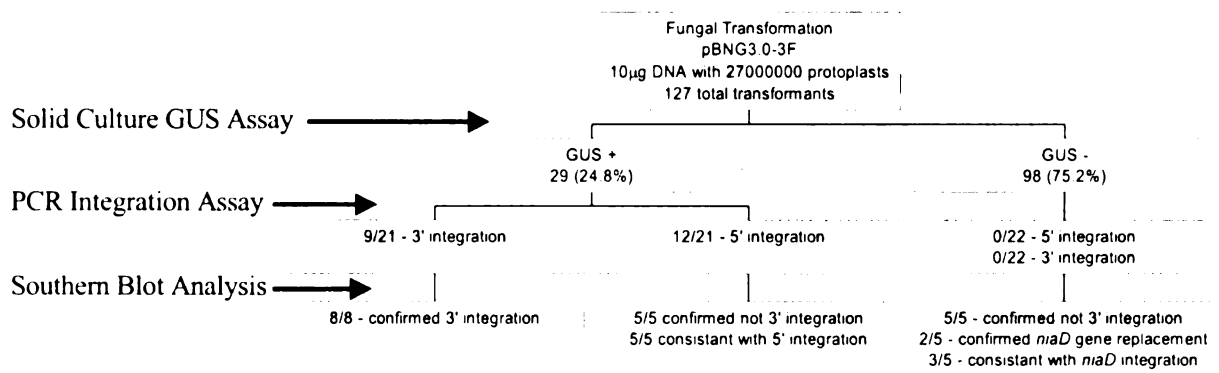


Figure 3.2. Experimental design. pBNG3.0-3F fungal transformants were first tested for GUS activity using a solid culture GUS assay. Selected GUS+ and GUS- transformants were then tested using a 3' and 5' *nor-1* site of integration PCR assay. Lastly, 3' *nor-1* integration status was confirmed for selected transformants using southern hybridization analysis

clearly evident for GUS positive colonies (Figure 3.3). 117 *niaD*⁺ transformants were initially tested for GUS activity by the solid culture assay. Of the 117 transformants tested, 29 were GUS positive colonies (24.8 %). In Figure 3.3, transformant 5 was GUS positive while transformants 1, 2, 3, 4 and 6 were GUS negative.

Genotype Screening of Transformants

In order to determine if the site of integration of pBNG3.0-3F is a factor in GUS activity, the chromosomal location of *nor-1::GUS* in 21 GUS positive and 22 GUS negative transformants was investigated. Each *nor-1::GUS* reporter construct could theoretically integrate into the *A. parasiticus* chromosome by homologous recombination at three independent sites: *niaD*, 5' *nor-1* (*nor-1* promoter) and 3' *nor-1* (*nor-1* terminator). Screening for clones in which integration occurred at the 3' *nor-1* locus is essential for two reasons: 1) *niaD* integrants have been shown to have lower transcriptional activity than aflatoxin cluster integrants (Liang et al., 1997 and Chiou et al., 2002); and 2) 5' integrants result in the chromosomal *nor-1* promoter fused to the GUS gene and the plasmid *nor-1* promoter fused to the chromosomal *nor-1* gene. A rapid site of integration PCR assay (Chiou et al., 2002) was initially used to screen these transformants. With the 3' *nor-1* PCR assay, a 2.1 kb fragment is diagnostic for 3' *nor-1* integration while a 3.1 kb fragment is diagnostic for 5' *nor-1* integration with the 5' *nor-1* PCR assay. All 21 GUS positive transformants were positive for 5' (12) or 3' (9) *nor-1* integration with the PCR assay (Figure 3.4). Conversely, 22 randomly selected GUS minus transformants were tested for site of integration using the rapid PCR assay and were all negative for 3' (Figure 3.5A) and 5' *nor-1* (Figure 3.5B) integration.

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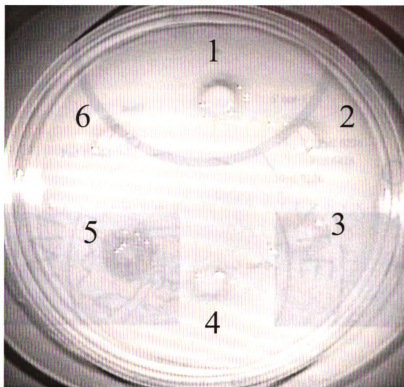


Figure 3.3. Solid culture GUS assay. Screening of transformants was performed using a solid culture GUS assay. Transformants 1, 2, 3, 4 and 6 are GUS- while transformant 5 is GUS+.

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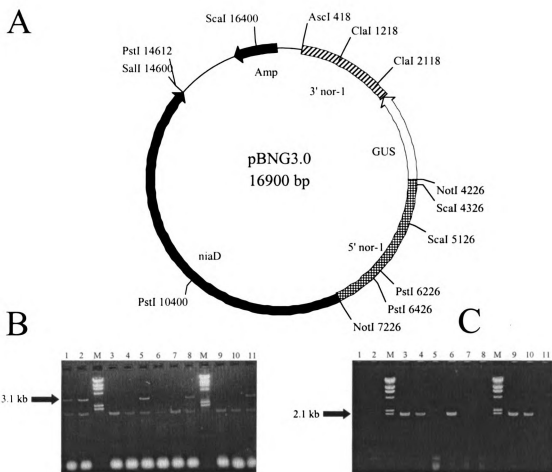
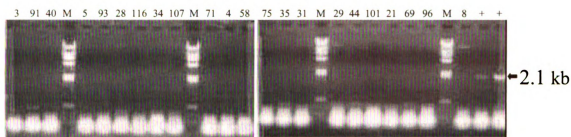


Figure 3.4. PCR analysis of site of integration in GUS+ transformants. (A) Restriction endonuclease map of pBNG3.0-3F. Only restriction endonuclease sites relevant to this study are shown. Ten GUS+ (lanes 1 to 6 and 8 to 11) and one GUS- transformant (lane 7) were analyzed for both 5' and 3' *nor-I* integration. Template DNA was prepared using a rapid boiling procedure. (B) Schematic for 5' *nor-I* integration with PCR data. 5' *nor-I* integrants resulted in a 3.1 kb PCR fragment (lanes 1, 2, 5, 8, 11). (C) Schematic for 3' *nor-I* integration with PCR data. 3' *nor-I* integrants resulted in a 2.1 kb PCR fragment (lanes 3, 4, 6, 9, 10). Lanes labeled M in panels B and C represent DNA ladder.

A



B

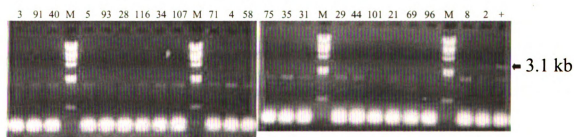


Figure 3.5. PCR analysis of site of integration in GUS- transformants. Template DNA was prepared using a rapid boiling procedure. (A) 3' site of integration PCR assay. All 22 randomly chosen (lane numbers indicate transformant number) GUS- transformants lacked the 2.1 kb PCR fragment diagnostic for 3' *nor-I* integration. Positive controls (lanes +) did contain the 2.1 kb PCR fragment. (B) 5' site of integration PCR assay. All 22 randomly chosen (same as in A) GUS- transformants lacked the 3.1 kb PCR fragment diagnostic for 5' *nor-I* integration. A positive control (lane +) did contain the 3.1 kb PCR fragment. All GUS- transformants were negative for both 3' (A) and 5' (B) *nor-I* integration. Lanes labeled M in panels B and C represent molecular size markers.

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While the PCR assay could identify 3' *nor-1* integrants, it could not identify multiple integrants (multiple 3' *nor-1* integrations and/or additional non-3' *nor-1* integrations). Consequently, Southern hybridization analysis was used to confirm PCR integration assay results for selected 3' *nor-1* (Figure 3.6A), 5' *nor-1* (Figure 3.6B) and non *nor-1* (Figure 3.6B) integrants. Disappearance of a 3.0 kb DNA fragment in the recipient strain, NR1, and the appearance of 3.7 and 4.0 DNA fragments is diagnostic for 3' *nor-1* integration. All PCR site of integration assay 3' *nor-1* integrants tested (8) were confirmed for 3' *nor-1* integration by Southern hybridization analysis (Figure 3.6A). While the Southern hybridization scheme utilized can not distinguish between 5' *nor-1*, *niaD* and heterologous integration, it can confirm that these transformants are not 3' *nor-1* integrants. All GUS+, 5' *nor-1* integrants analyzed were confirmed not to be 3' *nor-1* integrants (Figure 3.6B). All GUS negative transformants (also negative for 5' and 3' *nor-1* integration with the PCR assay) were also confirmed not to be 3' *nor-1* integrants (Figure 3.6B). In addition, two of these transformants (3 and 93) are likely products of double-crossover homologous recombination at *niaD* (gene replacement) because they contain only the 3.0 kb band seen in the recipient strain, NR1 (Figure 3.6B).

Solid culture GUS assay with 2 3' *nor-1* integrants, 2 5' *nor-1* integrants and 2 presumed *niaD* integrants is shown in Figure 3.7. The 3' and 5' *nor-1* integrants are GUS positive while the presumed *niaD* integrants are GUS negative. These data confirmed the data reported above for pAPGUSNN-B; ie integration at *nor-1* results in normal *nor-1* promoter activity while integration elsewhere results in undetectable levels of *nor-1* promoter activity. In addition, the rapid site of integration PCR assay is a useful tool to identify the valuable 3' *nor-1* integrants necessary for future promoter studies.

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Figure 3.6. Southern hybridization analysis of selected GUS+ and GUS- transformants for 3' *nor-1* integration. (A) Confirmation of 3' *nor-1* integration. All 8 transformants (designated by transformant number) were GUS+ and tested positive for 3' *nor-1* integration with the site of integration PCR assay. All 8 transformants tested confirmed 3' *nor-1* integration. (B) Testing for 3' *nor-1* integration. All 5 GUS- transformants (designated by transformant number) tested negative for 3' (and 5') *nor-1* integration using the site of integration PCR assay. Southern hybridization analysis confirmed lack of 3' *nor-1* integration in these 5 GUS- transformants. All 5 GUS+ transformants (designated by transformant number) tested positive for 5' *nor-1* integration with the site of integration PCR assay. Southern hybridization analysis confirmed lack of 3' *nor-1* integration in these 5 GUS+ transformants.

A

GUS+

NRI 14 19 32 43 67 68 81 78

4.0 →
3.7 →
3.0 →

B

GUS-

GUS+

NRI 3 91 40 5 93 2 22 23 26 41

4.5 →
3.0 →

Figure 3.6



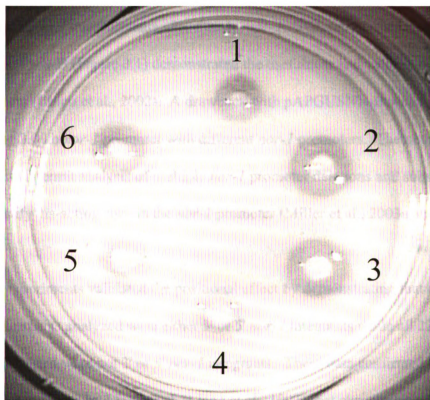


Figure 3.7. Solid culture GUS assay. Transformants 1 and 6 are 3' *nor-I* integrants and are GUS positive. Transformants 2 and 3 are 5' *nor-I* integrants and are GUS positive. Transformants 4 and 5 are not 3' or 5' *nor-I* integrants and are GUS negative.

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DISCUSSION

The goals of this study were (i) to generate a new *nor-1::GUS* reporter construct (pNANG-3) that enables easy promoter replacement and (ii) validate the positional-dependent regulation effect by correlating the site of integration with solid culture GUS activity for pBNG3.0 transformants. Previously, *nor-1::GUS* reporter strains generated using pAPGUSNN-B (Figure 3.1) demonstrated the usefulness and validity of the GUS reporter system (Chiou et al., 2002). A drawback with pAPGUSNN-B was the inability to replace the 3.0 kb *nor-1* promoter with different *nor-1* promoters. The new construct, pNANG-3, will permit analysis of multiple *nor-1* promoter deletions and substitutions in order to identify *cis*-acting sites in the *nor-1* promoter (Miller et al., 2003a and Miller et al., 2003b).

Our experiments validated the positional effect by demonstrating that all 21 GUS+ transformants analyzed were either 3' or 5' *nor-1* integrants while all 22 GUS- transformants tested were not 3' or 5' *nor-1* integrants. These data confirmed the preliminary observation by Liang et al. (Liang et al., 1997) that chromosome location plays a role in regulation of aflatoxin gene expression. The targeting of reporter constructs to specific chromosomal locations for promoter analysis has been suggested by other investigators studying expression of fungal genes (Hamer and Timberlake, 1987 and Timberlake and Marshal, 1988 and van Gorcom et al., 1986). Although the mechanisms of position-dependent gene expression have not been fully elucidated in filamentous fungi, it has been hypothesized that enhancer elements may be responsible for the position-dependent effect (Kinsey and Rambosek, 1984). The hypothesis that positive *cis*-acting factors have regional control over the transcription of aflatoxin genes

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is reasonable, since removal of aflatoxin reporter fusions from the aflatoxin gene cluster results in reduced GUS expression.

An alternative explanation for the lack of GUS activity at the *niaD* loci is that a transcriptionally inactive chromatin structure exists at this site. We think that such inactivation is unlikely for two reasons. First, NR1 transformed with pGAPN2B (β -tubulin::GUS) expressed GUS activity at similar levels at 24, 48, and 72 h when integrated at the *niaD* locus indicating that transcriptional activation can occur at the *niaD* locus in YES agar (Chiou et al., 2002). Second, in transformants carrying pAPGUSNP (*nor-1*::GUS with *pyrG* as selectable marker), the *nor-1* promoter carried on this plasmid was not active at the *pyrG* locus even though growth on YES medium required expression of the *pyrG* gene (Chiou et al., 2002). In addition, several pAPGUSNP transformants had no detectable GUS activity even when they carried multiple copies of plasmid as long as no copies of the plasmid integrated at 5' or 3' *nor-1* (Chiou et al., 2002). Based on previous data (Liang et al., 1997 and Chiou et al., 2002) and the data presented here, we hypothesize that aflatoxin gene expression is influenced by an locus control element.

The clustering, or close linking, of genes involved in the same secondary metabolic pathway is a common theme in the filamentous fungi (Keller and Hohn, 1997). However, *cis*-acting elements that regulate many genes simultaneously in fungal gene clusters have not been identified. If locus control region does influence the regulation of *nor-1* transcription, it is located at least 3 kb upstream from the transcription initiation site in the 5' *nor-1* region, at least 1.8 kb downstream of the transcription termination site in the 3' region of *nor-1*, or within the *nor-1* coding region. Because of similar position-dependent expression observed with the *ver-1*::GUS reporter construct (Liang et al.,

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1997), it is also possible that the same locus control region is influencing the regulation of both *nor-1* and *ver-1*. Studies performed on the SpoC1 gene cluster in *A. nidulans* showed that clustered genes can be coordinately expressed during development and that placement of cluster genes at ectopic chromosomal locations results in the loss of that coordination (Miller et al., 1987 and Timberlake and Barnard, 1981). The physical linkage of aflatoxin biosynthesis genes also may have a regulatory role. Trichothecene biosynthesis genes are also clustered (Hohn et al., 1993b) and there is some evidence for positional-dependent regulation with the pathway regulator Tri6 (Chen et al., 2000). Introduction of a plasmid containing a Tri5::GUS fusion with a functional Tri6 results in 50-100X more GUS activity with Tri5 integration compared to ectopic integration (Chen et al., 2000). Studies designed to establish such a phenomenon and to determine the nature of the regulatory mechanism may eventually lead to a broader understanding of the expression of secondary metabolism genes and the ability to manipulate its expression in microorganisms.

We utilized a rapid procedure to prepare DNA templates from fungal colonies that are sufficiently pure for the site of integration PCR assay. This development was critical for current and future studies on the regulation of the *nor-1* and *ver-1* promoters because detection of the correct site of integration in the genome is essential to accurately measure environmental influences on the aflatoxin gene promoters. In this regard, the speed and effectiveness of the rapid assay make screening of large numbers of fungal transformants practical. In addition, screening of transformants can be based on genotype (site of integration) rather than phenotype (GUS activity) which is important for *nor-1*::GUS constructs that have reduced GUS activity.

ACKNOWLEDGMENTS

The work in Chapter 3 was a contribution to a publication by Ching-Hsun Chiou (Chiou CH, Miller MJ, Wilson DL, Trail F and Linz JE. 2002. Chromosomal location plays a role in regulation of aflatoxin gene expression in *Aspergillus parasiticus*. *Applied and Environmental Microbiology*. 68(1): 306-315).

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CHAPTER 4

Role of AflR in *nor-1* transcriptional activation in *Aspergillus flavus* and *A. parasiticus*

INTRODUCTION

Aflatoxins, mycotoxins produced predominantly by *Aspergillus parasiticus* and *A. flavus*, frequently contaminate several economically important crops including corn, cotton, peanuts and tree nuts (Wilson and Payne, 1994). Aflatoxin B₁ (AFB₁), the most abundant of the aflatoxins, is also the most toxic and carcinogenic (McLean and Dutton, 1995). Animal studies have shown aflatoxin to be a potent hepatocarcinogen and human epidemiological data have linked aflatoxin exposure with liver cancer (reviewed in Hall and Wild, 1994 and Dvorackova, 1990). As a result, crops susceptible to aflatoxin contamination are monitored for aflatoxin concentrations in the United States and throughout the world imparting a huge economic cost to growers and marketers of commodities. Consequently, our long-term goal is to reduce or eliminate aflatoxin from the food chain.

To accomplish this goal, we are focused on elucidating the molecular mechanisms that regulate aflatoxin biosynthesis. This information is likely to generate novel approaches and targets for inhibition of aflatoxin gene expression. Aflatoxin biosynthesis is a complex process that requires at least 16 different enzymatic steps (Bhatnagar et al., 1994). The genes involved in aflatoxin biosynthesis reside in a 70 kb cluster and appear to be co-regulated (Trail et al., 1995b). Analysis of the regulatory mutant *A. flavus* strain 650 (Bennett and Papa, 1988) first identified AflR as a pathway

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regulator (Payne et al., 1993). Subsequently, *aflR* homologs were identified in *A. parasiticus* (Chang et al., 1993) and *A. nidulans* (Yu et al., 1996). Several pieces of evidence demonstrate a key regulatory role for AflR in aflatoxin biosynthesis: 1) based on amino acid sequence identity, AflR belongs to a common class of fungal transcription factors called zinc binuclear cluster proteins (Woloshuk et al., 1994); 2) *aflR* null mutants do not produce detectable aflatoxin biosynthetic enzymatic activities (Payne et al., 1993) nor the aflatoxin biosynthetic transcripts *stcS*, *stcV*, *stcU*, *stcT* (Yu et al., 1996); 3) increased transcription of *aflR* is correlated with increased aflatoxin production (Chang et al., 1993, Yu et al., 1996, Flaherty and Payne, 1997); 4) Recombinant AflR binds to several aflatoxin biosynthetic gene promoters *in vitro* (Fernandes et al., 1998, Ehrlich et al., 1999a and Ehrlich et al., 1999b); and 5) AflR *cis*-acting sites have been shown to be necessary for transcriptional activation for three aflatoxin biosynthetic genes *in vivo* including *stcU* (Fernandes et al., 1998), *avnA* (Ehrlich et al., 2002) and *pksA* (Cary et al., 2000). Although it is clear that AflR is a key regulator of aflatoxin synthesis, it is not clear if AflR is the only specific transcription factor needed for transcriptional activation of all aflatoxin biosynthesis genes and if all consensus AflR *cis*-acting sites in the aflatoxin cluster are functionally significant.

To address these questions, we focused attention on expression of the *nor-1* gene that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin (Trail et al., 1994 and Zhou and Linz, 1999). The *nor-1* promoter includes a consensus AflR binding site (TCGnnnnnCGR) located at -75 to -64 bp (AflR1) from the primary *nor-1* transcriptional start site (+1); additional upstream consensus AflR binding sites are located at -1213 (AflR2) and -1563 (AflR3). Genes upstream from *nor-1* include an open reading frame of unknown function (ORF3:

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translational start at -1073) and the divergently transcribed *pksA* (translational start at -1731) (Trail et al. 1995, Chang et al. 1996).

A. parasiticus nuclear extracts and recombinant AfIR were unable to bind to a DNA fragment containing AfIR1 *in vitro* (Ehrlich et al., 1999b) suggesting that AfIR1 might not be functionally relevant. Directed mutations in AfIR1 did not affect transcription of the divergently transcribed *pksA in vivo* (Ehrlich et al., 2002) suggesting AfIR1 may not be important for expression of *pksA* (effects of AfIR1 mutation on *nor-1* or ORF3 expression were not reported). However, using the same mutagenesis approach, both AfIR2 and AfIR3 were shown to be necessary for *pksA* transcription but effects of AfIR2 and AfIR3 mutation on *nor-1* and ORF3 expression were not reported (Ehrlich et al., 2002). Furthermore, alteration of additional *cis*-acting sites by directed mutagenesis (BrlA and PacC), significantly altered *pksA* transcription indicating that AfIR may not be the only transcriptional regulator for *pksA* (Ehrlich et al., 2002). In contrast, other experimental data suggest that AfIR alone is necessary for transcriptional activation of the middle pathway gene *avnA* through a single consensus *cis*-acting site (Cary et al., 2000). An additional potential AfIR binding site (TCGnnnnnCGR) in the *avnA* promoter, when mutated, did not alter *avnA* transcription *in vivo* and recombinant AfIR protein was unable to bind to this non-functional binding site *in vitro* (Cary et al., 2000). The *stcU* promoter has three consensus AfIR binding sites within 800 bp of the translational start point (Fernandes, et al., 1998). Directed mutations in the three AfIR sites revealed that the most distal site, -762, had no effect on *stcU* transcription while AfIR consensus binding sites at -81 and -168 both appeared to be functional *in vivo* (Fernandes et al., 1998). The two functional AfIR binding sites were not additive because *stcU* promoters with only the -81 site or the -168 site were indistinguishable from strains that had both

AflR binding sites (Fernandes et al., 1998).

The objective of the current study was to clarify a role for AflR and the consensus AflR binding sites AflR1, 2 and 3 in expression of *nor-1* in the predominant aflatoxigenic species *A. flavus* and *A. parasiticus*. More specifically, we wanted to: 1) analyze AflR binding to AflR1 in the *nor-1* promoter in *A. flavus in vitro*, 2) determine if AflR1 is necessary for *nor-1* transcriptional activation in *A. parasiticus* and *A. flavus in vivo*, 3) determine if AflR2 and AflR3 are necessary for *nor-1* transcriptional activation in *A. parasiticus in vivo* and 4) determine if AflR is the only transcriptional activator necessary for *nor-1* expression in *A. parasiticus*. Although the relevant AflR consensus sites were identified previously based on sequence analysis (Ehrlich et al., 2002), this was the first functional analysis of these sites with respect to *nor-1* promoter function in *A. parasiticus* and *A. flavus*. We showed that recombinant *A. flavus* AflR bound to an oligonucleotide containing AflR1 using the Southwestern blot procedure. Additionally, deletion analysis of the *nor-1* promoter in *nor-1::GUS* reporter strains showed that AflR1 is necessary for *nor-1* transcriptional activation in *A. flavus in vivo*. Using analogous *nor-1::GUS* reporter strains in *A. parasiticus* containing *nor-1* promoter deletions and substitutions, we demonstrated that AflR1 and possibly AflR2 and additional *cis*-acting site(s) are necessary for *nor-1* transcriptional activation *in vivo*. We also tentatively identified a novel gene (ORF3) that may function to directly or indirectly activate *nor-1* transcription in *A. parasiticus*.

MATERIALS AND METHODS

Strains

Aspergillus parasiticus NR-1 (*niaD*; [Hornig et al., 1990] and *A. flavus* 656-2 (white, *aflR*, *pyrG*, *leu*; [Payne et al., 1993]) were used as recipient strains for transformations. *Aspergillus flavus* NRRL 3357 (National Center for Agricultural Utilization Research, Peoria IL) is a wild type strain.

Plasmid Construction

A. parasiticus reporter plasmids

The plasmids pNANG-1 and pBNG-3.0 have been previously described (Chiou et al., 2002). Additional *nor-1* promoter pieces were generated by PCR using pAPGUSNN-B as template with primers with *NotI* (3') and *PacI* (5') tails. The PCR products were ligated into the *NotI* and *PacI* sites in pNANG-3 resulting in pBNG-n (where n designates the size of the promoter in base pairs). Plasmid maps for pNANG-3 and pBNG-3.0 are shown in Figure 4.1.

A. flavus reporter plasmids

GAP4 contains a promoterless *E. coli uidA* (GUS) gene (Woloshuk and Payne, 1994). A *BamHI* site was introduced at the *nor-1* translational start site by site directed mutagenesis (Kunkel, 1985). Then a 1.3 kb *BamHI* fragment containing the *nor-1* promoter was ligated into the *BamHI* site in GAP4 resulting in GAP12. The *nor-1::GUS* reporter plasmids GAP12-138, GAP12-103 and GAP12-91 were created by PCR using GAP12 as a template. The downstream primer for all three constructs was the M13F

Figure 4.1. Restriction endonuclease maps of relevant plasmids. (A) pNANG-3. In addition to the *niaD* selectable marker, pNANG-3 carries a small part of the *nor-1* coding sequence (10 amino acids) fused to the β -glucuronidase (GUS) coding sequence which is in turn fused to the 2 kb *nor-1* 3' terminator fragment. Appropriate promoter pieces, amplified by PCR using primers with *NotI* and *PacI* tails, were cloned into pNANG-3. The small number of codons that were changed in the *nor-1* coding sequence to create the useful *NotI* site were all acceptable based on codon usage and maintained the correct sense. (B) pBNG3.0-3F. This plasmid contains a 3 kb PCR amplified *nor-1* promoter piece cloned into the *NotI* site of pNANG-3. Other *nor-1* promoters that were tested (1250, 1200, 664, 332P, 332, 332AflRmut, 76 and 64) were also made by PCR and were directionally cloned into the *NotI* and *PacI* sites in pNANG-3. (C) pGAP12. This plasmid contains the GUS gene connected to a 1.3 kb *BamHI* *nor-1* promoter fragment. pGAP12 was used as a template for PCR to make the *A. flavus* reporter plasmids used in this study. (D) pGAP12-138. This plasmid contains 138 bp upstream of translational start site of *nor-1*.

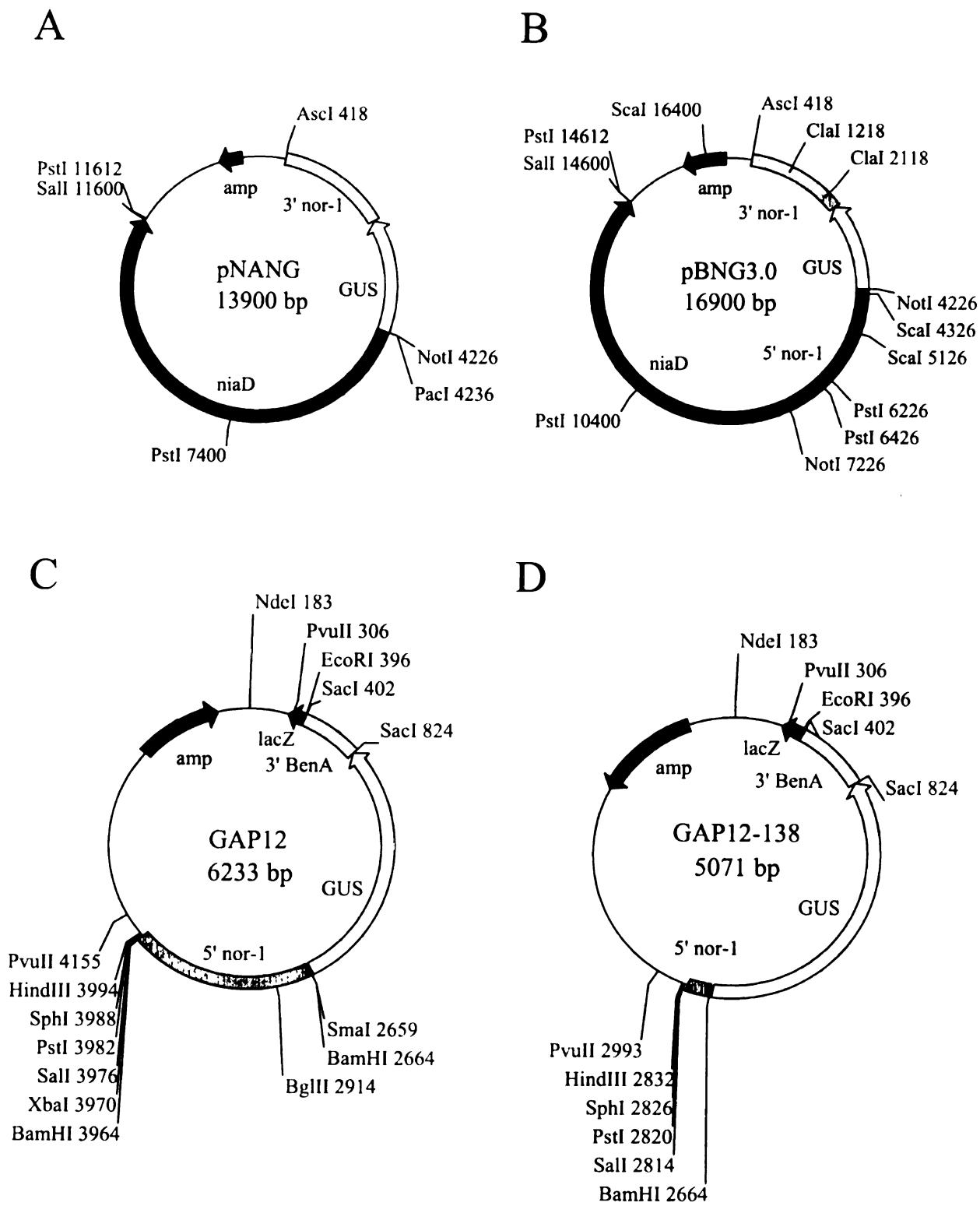


Figure 4.1

primer. The upstream primers were as follows: GTTGGCATAACCATCAAATGC for GAP12-138, CCAACTCGGCCAGCGACCAACACACCACC for GAP12-103 and GCGACCAACACACCACC for GAP12-91. The PCR product was cloned into the pCRII vector from the Invitrogen TA Cloning Kit (Invitrogen, Carlsbad CA). Plasmids were named GAP12-n where n designates the number of bases upstream from the *nor-1* translational start site included in the vector. Figure 4.1 includes a map of GAP12 and GAP12-138.

A. flavus tAflR expression plasmid construction

The open reading frame (ORF) of *aflR* was amplified from the cosmid B9X2 (Flaherty, et al., 1995) by PCR with primers that also contained restriction enzyme sites for ease of cloning the product in-frame into the expression vector, pET30c (Novagen, Madison WI). The upper (5') primer (5'-GCGGATCCAAATGGTGACCATATCTCCC C-3') contained a restriction site for the endonuclease *Bam*HI (bolded) and 20 nucleotides of *aflR* sequence (underlined), beginning at the start codon of the open reading frame. The lower (3') primer (5'-GCGAAGCTTATAATGTCGGAGGACACGGCG-3') contained a restriction site for the endonuclease *Hind*III (bolded) and 21 nucleotides of *aflR* sequence (underlined) from the 3' end of the ORF up to, but not including, the stop codon. The resulting PCR product was 1330 bp in length (the *aflR* open reading frame is 1311 bp in length). This product was cloned into the pCRII vector from Invitrogen (San Diego, CA) and the resulting plasmid was subsequently digested with *Bam*HI and *Hind*III restriction enzymes for isolation of a 1323 bp fragment. This fragment was then cloned into the pET30c expression vector at the *Bam*HI and *Hind*III sites. The resulting expression construct, pET30c-AFLR, contains a 1503 bp ORF, encoding 501 amino

acids. The predicted protein product has a molecular mass of 53,940 Daltons. The *aflR* ORF makes up 437 of the 501 amino acids. The other 64 amino acids make up the histidine tags at the N-terminal and C-terminal ends and an S-tag and enterokinase cleavage site at the N-terminal end of the recombinant AFLR protein.

Because of solubility problems with the full-length AFLR recombinant protein, a truncated AFLR protein that contained the DNA binding domain was desired. To produce the pET30c-tAFLR construct for overexpression of a truncated AFLR protein, the pET30c-AFLR construct was digested with *XhoI* and *HinDIII* and then religated. This allowed retention of 665 bp of the original 1323-bp fragment that was cloned into the pET30c vector. This 665-bp fragment contains sequence for the N-terminal histidine tag, the S-tag, the enterokinase cleavage site, and the 5' end of the *aflR* ORF including the entire AFLR zinc binuclear cluster region, but the 3' end of the *aflR* ORF was deleted.

Generation and Selection of Transformants

Transformation

Transformation of *A. parasiticus* protoplasts was performed as described by Horng et al. (1990). 2-4 µg of DNA was used with approximately 10⁷ protoplasts resulting in approximately 100 transformants. Transformation of *A. flavus* was as described by Woloshuk (Woloshuk et al., 1989).

Site of Integration PCR Assay (*A. parasiticus*)

A rapid DNA extraction procedure and PCR analysis were performed as

described by Chiou (Chiou et al., 2002). Following PCR, a 2.0 kb DNA fragment is diagnostic for 3' integration.

Southern Hybridization (*A. parasiticus*)

Genomic DNA was purified from *A. parasiticus* cultures shaken for 48 h in 100 ml YES (2% yeast extract, 6% sucrose and pH=5.8) liquid medium at 29°C with five, 6 mm glass beads (Skory et al., 1993) and subjected to standard agarose gel electrophoresis (Ausubel et al., 2003). Southern hybridization analysis was performed according to standard procedures (Ausubel et al., 2003). 2.5 µg of genomic DNA was digested with *ScaI* and probed with a 900 bp *Clal* fragment isolated from the *nor-1* terminator region of pAPGUSNN-B (Chiou et al., 2002). Digestion of DNA from the recipient strain NR-1 generates a 3.0 kb restriction fragment while a 3' integrant results in 3.7 and 4.0 kb restriction fragments.

Dot Blot Hybridization Analysis (*A. flavus*)

Co-transformants were first screened for uracil prototrophy and then screened for presence of the reporter plasmid by dot blot hybridization of genomic DNA with a GUS gene probe. Genomic DNA from cotransformants was isolated as previously described (Woloshuk, et al., 1989). Each genomic DNA sample was boiled in a total volume of 0.5 ml (final concentration = 0.4 M NaOH and 10 mM EDTA) for 10 minutes. The samples were applied to Zeta probe nylon membranes (BioRad, Melville NY) on a dot blot apparatus (Schleicher and Schuell, Keene NH) per manufacturers' instructions. The loaded membranes were rinsed in 2X SSPE (0.18 M NaCl, 10 mM NaPO₄ (pH 7.7), 1 mM EDTA), air dried, and then cross-linked. The cross-linked membranes were probed

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with a 2.0 kb *KpnI* fragment of the *E. coli uidA* gene from GAP13 (Flaherty et al., 1995) for selection of primary cotransformants containing the GUS gene.

GUS Reporter Assays

Solid culture GUS Assay (*A. parasiticus*)

Autoclaved Nytran SPC (Schleicher & Schuell, Keene NH) membranes were first placed on top of YES (2% yeast extract, 6% sucrose, 1.5% agar and pH=5.8) agar plates. Transformants were then transferred via sterile toothpick to the Nytran membrane. After incubation for 46 h at 29°C in the dark, the Nytran filters were removed, frozen in liquid nitrogen and thawed at room temperature (2 repetitions) and then incubated for up to 24 h with GUS substrate solution that includes 0.04% of the colorimetric GUS substrate (X-Glu: 5-bromo-4-chloro-3-indolyl β -D-glucuronide) in GUS reaction buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 0.07% β -mercaptoethanol) and visually evaluated.

Liquid Culture GUS Assay (*A. parasiticus*)

Two confirmed *A. parasiticus* 3' transformants containing reporter constructs were grown in duplicate for 48 h at 29°C in the dark with shaking in 100 ml of GMS (Buchanan and Lewis, 1984) liquid medium in a 250 ml flask with five 6 mm glass beads. Mycelia were collected by filtration through Miracloth (Calbiochem, La Jolla CA) and ground in a mortar with a pestle under liquid N_2 . Approximately 200 mg of ground mycelia was transferred to a 1.5 mL microcentrifuge tube and kept on ice. After all the samples had been collected, 500 μL of GUS lysis buffer (50 mM NaH_2PO_4 [pH=7], 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 0.07% β -mercaptoethanol and 25

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mg/ml PMSF [phenylmethylsulfonyl fluoride]) was added. The samples were vortexed for 15 s and then centrifuged for 10 min at 10,000 X g at 4°C. The supernatant was withdrawn and placed in a new tube. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules CA). GUS activity was determined with 100 µg of each sample incubated at 30°C with 145 µg of the GUS substrate 4-methylumbelliferyl β-D-glucoside (MUG) in a final volume of 200 µl. At 0, 10 and 30 min, 800 µl of stop solution (200 mM Na₂CO₃) was added. After stopping the GUS reaction, 200 µl of sample was loaded into a microtiter plate which was analyzed using a Cytofluor II fluorometer (Biosearch Co., Bedford MA). Excitation and emission wavelengths were 360 nm and 460 nm, respectively. To evaluate the fluorometer results, a standard curve of 4-Methylumbelliferone (10 nM to 600 nM) in GUS lysis buffer was also read in the fluorometer. GUS activity was reported as pmols 4-methylumbelliferone / minute / mg protein.

Quantitative GUS Assay (*A. flavus*)

Growth of selected transformants for quantitative analysis of GUS activity was carried out in 24-well plates (Becton Dickinson & Company, Lincoln Park, NJ) in media conducive (sucrose low salts; SLS) and non-conducive (peptone mineral salts; PMS) to aflatoxin production and expression of pathway genes, as previously described (Flaherty, et al., 1995). Conidia (1x10⁶/well) were suspended initially in 1 ml of PMS and allowed to grow for 3 d at 28°C. After this initial growth period, three replicate mycelial mats of each transformant were collected (0 h time point), while three additional replicate mycelial mats of each transformant were resuspended in 1 ml of either PMS or SLS medium and allowed to continue growth at 28°C. These additional replicates were

harvested 24 h after resuspension (24 h PMS and 24 h SLS timepoints). The harvested mycelial mats were placed in 1.5 ml disposable snap-cap tubes, quick-frozen with liquid N₂, and placed at -80°C until protein extraction could be carried out. Cultures for each time point (0 h, 24 h PMS, and 24 h SLS) were grown in separate 24-well plates, and after collection of the mycelial mats at each timepoint, the plates were placed at -20°C to preserve the culture filtrates for aflatoxin analysis. The GUS activity of cotransformants was measured quantitatively using the procedures of Jefferson (Jefferson, 1987) for analyzing GUS activity in plant tissue as modified by Flaherty (Flaherty et al., 1995) for analyzing GUS activity in *A. flavus* tissue. One exception was that 50 µl of each extracted fungal protein sample was added to 500 µl of GUS substrate buffer (1 mM 4-methyl-umbelliferyl β-D-glucuronide in extraction buffer) which had been pre-incubated at 37°C. After 1 h incubation at 37°C, 100 µl of assay buffer (extracted protein sample + substrate buffer) was removed and added to 900 µl of stop buffer (0.2 M Na₂CO₃). Relative fluorescence was subsequently measured using a Hoefer TK-100 fluoremeter following the manufacturer's instructions. GUS activity was reported as nmole MU / min / mg protein.

A. flavus tAflR (truncated AflR) binding studies

Induction and Purification of tAflR

A truncated *aflR* gene product, tAFLR, was expressed in a bacterial system for production and purification of recombinant tAFLR protein for use in DNA binding studies. Single colonies of PET30c-tAflR were used to inoculate LB-Kan (30µg/ml, 500 ml LB). The culture was grown at room temperature for 4 h followed by 37°C for 3 h with shaking at 200 rpm. When the OD₆₀₀ was approximately 0.9, IPTG (isopropyl-β-D-

thiogalactopyranoside) was added to a final concentration of 0.4mM. Cultures were allowed to grow for an additional 2-3 h. Cells were collected by centrifugation and frozen for subsequent purification. A pellet from 250ml of culture was used for purification on a Ni-NTA agarose column (Qiagen, Valencia CA) according to manufacturers specifications with modifications. Briefly, the pellet was resuspended in 4 ml Buffer B (8M urea, 0.1M NaH₂PO₄, 0.01M TRIS/HCL, pH 8.0). Cells were partially lysed by gentle homogenization and centrifuged to obtain a cleared lysate (10,000 x g for 10 min.). The Ni-NTA column was pre-equilibrated with Buffer B and 1.8 ml cleared lysate was added to the column. The column was washed with 9 ml Buffer C (8M urea, 0.1M NaH₂PO₄, 0.01M TRIS/HCL, pH 6.3). After washing, the protein was eluted with 9 ml Buffer E (8M urea, 0.1M NaH₂PO₄, 0.01M TRIS/HCL. PH 4.5). A final elution step was performed with 3 ml of 0.75 x Buffer E with 250mM imidazole.

The final 6 ml of the eluted fractions were dialyzed in 500ml dialysis buffer (10mM TRIS, pH7.6, 200mM KCL, 1mM EDTA, 5mM DTT and 10% glycerol) with decreasing concentrations of urea : 6M, 3M, 2M, 0.5M and 0M.. Protein concentration was determined by the Bradford method.

Preparation of oligo probes

PP2 was used a probe while PP2 and PP2MUT were used as competitors for Southwestern blot analysis. PP2 (CCA**ACTCGGCCAGCGACCAACACACC**ACC) sequence is from the *A. flavus nor-1* promoter and includes AfIR1, an AfIR consensus binding site (TCGnnnnnCGA). PP2MUT (CCAACGCTGCCAGCGACCAACACACC) is the same as PP2 except for two mutations (underlined residues) in the consensus AfIR binding site. Double stranded oligonucleotides were used for labeling

and cold competition experiments. A typical end-labeling reaction used 1 pmole double stranded oligo with 8-10 units polynucleotide kinase (Promega, Madison WI) and 1.5 μ l gamma ATP (3000 Ci/mmol, 10 mCi/ml) (New England Nuclear, Boston MA). The reaction was incubated at 37°C for 10 minutes and stopped with the addition of 2 μ l 0.5 M EDTA. Oligonucleotides were purified with Centri Spin 20 columns (Princeton Separations, Adelphia NJ) according to manufacturers instructions.

Southwestern Blot

Southwestern blots were performed essentially as described by Tully and Cidlowski (Tully and Cidlowski, 1993). Briefly, 2.8 μ g of tAflR per lane was fractionated on SDS-PAGE, incubated for 2 x 1h washes in renaturation buffer (50mM NaCl, 10mM TRIS-HCl at pH 7.5, 20mM EDTA, 0.1mM dithiothreitol and 4M urea) and electro-blotted (mini tank system from BioRad, 84volts and initially 294 milliamps, 1-1.5 h) onto nitrocellulose. Nitrocellulose filters were blotted overnight in 200ml blocking buffer (5% (w/v) nonfat dry milk, 50mM NaCl, 10mM TRSI-HCl, pH 7.5, and 1mM EDTA). Vertical strips, containing a molecular weight marker lane and a tAflR lane, were cut from the nitrocellulose filter. Filters were placed into heat-sealable plastic bags and incubated for 1.5 h at room temperature with 1 ml blocking buffer or 1 ml blocking buffer supplemented with 5 pmole competitor DNA. 250 ml blocking buffer containing 2×10^6 cpm PP2 (approx. 0.34 pmole for a specific activity of 1×10^8 cpm/mg) was added to each bag and incubated at room temperature for 1.5h. Filters were removed from the bag and washed in 50 ml blocking buffer for 30 min, rinsed briefly in TBS-T, allowed to dry and autoradiography was performed.

RESULTS

AflR binding to the *A. flavus nor-1* promoter

Southwestern blot analysis was performed to test the ability of a truncated AflR (tAflR) to bind to an oligonucleotide containing an AflR consensus binding site (AflR1, Figure 4.2). ³²P labeled oligonucleotide PP2 (contains AflR1 binding site from the *A. flavus nor-1* promoter) bound to tAflR (lane 1). To demonstrate specificity, competition experiments were performed. A 15 fold molar excess of unlabeled PP2 competed with labeled PP2 (lane 2) while a 15 fold excess of PP2MUT (carries two mutations in the consensus AflR binding site) competed marginally with labeled PP2 for tAflR binding (lane 3). These data confirm that AflR protein can bind to AflR1 in a specific manner.

A. flavus GUS Activity Assays

Aspergillus flavus 656-2 was co-transformed with plasmid pAF-1 containing the *pyr4* gene for uracil biosynthesis (Payne et al., 1993) and GAP12-n (n = size of *nor-1* promoter). Dot blot analysis was used to screen the *pyr4+* transformants for presence of the GUS gene (data not shown). Five transformants of each deletion construct that contained a single copy of GUS were used for quantitative analysis. Each transformant was grown for 3 days in PMS (aflatoxin non-supportive medium) and then transferred to either PMS or SLS (aflatoxin supportive medium) for 1 d. Three different *A. flavus* strains (see Figure 4.3) were used for GUS analysis. GAP12-91 (lacks AflR1) had no detectable GUS activity in either PMS (non aflatoxin supportive medium) or SLS (aflatoxin supportive medium). GAP12-103 and GAP12-138 both contain AflR1 and had GUS activity in PMS and SLS. However, a 4.5 fold induction (activity in SLS divided

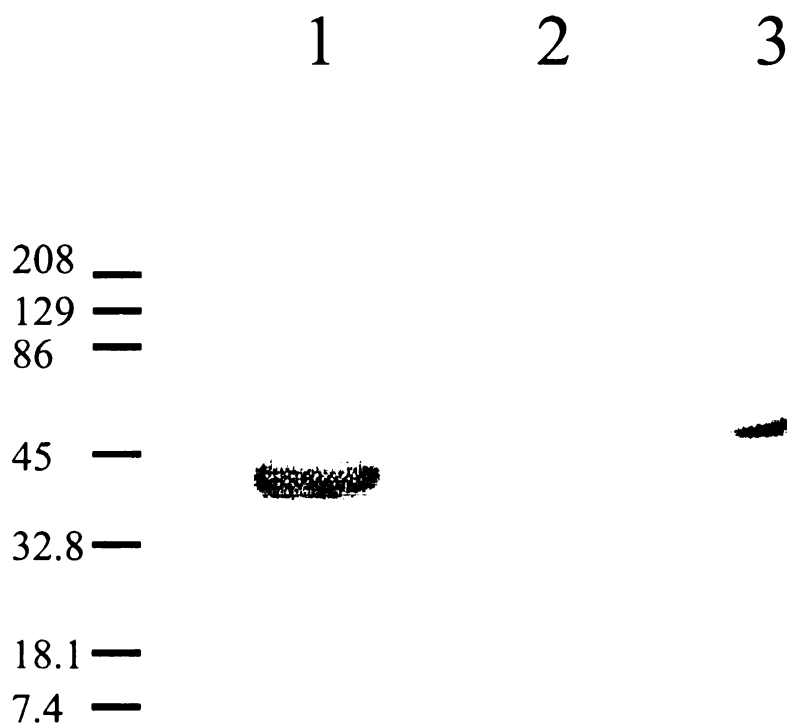


Figure 4.2. Southwestern blot analysis of AflR1 with purified tAflR (*A. flavus*) and a PP2 probe. PP2 (CCA**ACTCGGCCAGCGACCAACACACCACC**), a 29 bp oligo from the *A. flavus nor-1* promoter, contains the AflR binding site AflR1 (bold). PP2MUT (CCAACGCTIGCCAGCGACCAACACACCACC) is the same as PP2 except for 2 mutations (underlined) in the AflR binding site. PP2 was labeled with ^{32}P and used as probe in all three lanes. Competitors had a 15 fold molar excess and were unlabeled. Lane 1, no competitor; lane 2, PP2 competitor; lane 3, PP2MUT competitor

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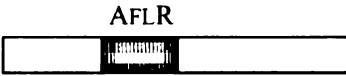
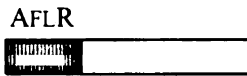
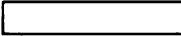
		<u>PMS</u>	<u>SLS</u>	<u>Fold Induction</u>
GAP 12-138		2.2	9.9	4.5
GAP 12-103		3.5	9.9	2.8
GAP 12-91		N.D.	N.D.	-

Figure 4.3. Schematic of the *A. flavus nor-1* promoters and GUS analysis. The AfIR binding site AfIR1 is located between residues -103 and -91. Fold induction was determined by dividing the GUS activity (nmole MU / min / mg protein) in an inductive medium (SLS) by GUS activity in a non-inductive medium (PMS).

by activity in PMS) was observed for GAP 12-138 and a 2.8 fold induction was observed for GAP 12-103 confirming that AflR1 is necessary for *nor-1* transcriptional activation in *A. flavus in vivo*.

Generation and Screening of *A. parasiticus* Transformants

In order to determine whether AflR is necessary for *nor-1* transcriptional activation in *A. parasiticus*, several *nor-1::GUS* reporter plasmids were constructed (Figure 4.4). We defined the upstream border of the *nor-1* promoter as the polyadenylation site for ORF3, located 332 bp upstream from the primary transcriptional start site of *nor-1*. Deletion analysis generated clones with 332, 76 and 64 bp promoter fragments (AflR1 located between -76 and -64) carried on *nor-1::GUS* constructs. GUS activity in transformants that carry these plasmids was analyzed to determine functionality of AflR1. In addition, a transformant carrying a substitution of AflR1 (TCGgccagCGR to AGTttaaCAG) in the context of the 332 bp promoter was also analyzed. Clones carrying larger *nor-1* promoter fragments (3000, 1250, 1200 and 664 bp) were analyzed to determine if any additional upstream *cis*-acting sites including AflR2 and AflR3 were involved in *nor-1* transcriptional activation.

Each *nor-1::GUS* reporter construct could theoretically integrate into the *A. parasiticus* chromosome by homologous recombination at three independent sites: *niaD*, 5' *nor-1* (*nor-1* promoter) and 3' *nor-1* (*nor-1* terminator). Screening for clones in which integration occurred at the 3' *nor-1* locus was essential for two reasons: 1) *niaD* integrants have been shown to have lower transcriptional activity than aflatoxin cluster integrants (Liang et al., 1997 and Chiou et al., 2002); and 2) 5' integrants result in the chromosomal *nor-1* promoter fused to the GUS gene and the plasmid *nor-1* promoter

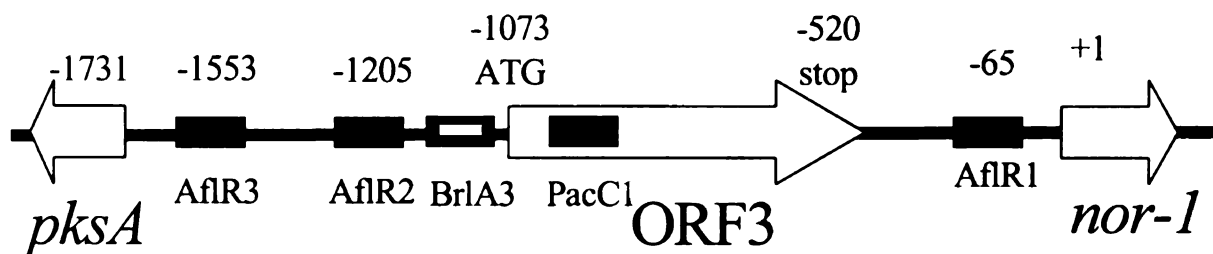


Figure 4.4. Schematic of the *nor-1* promoter region in *A. parasiticus*. The numbers indicate the number of nucleotides included upstream from the transcriptional start site. Several potential *cis*-acting sites are indicated including the AflR binding sites AflR1, AflR2 and AflR3 and PacC1 and BrlA3 which were reported to be involved in *pksA* transcriptional regulation (Ehrlich et al., 2002). The location of an open reading frame (ORF) of unknown function is also shown. The sizes of the *nor-1* promoters used in this study are also indicated (1250, 1200, 664, 332, 76 and 64).

fused to the chromosomal *nor-1* gene. Transformants from the *nor-1::GUS* reporter constructs were screened initially by a rapid PCR assay specific for *nor-1*, 3' integration (Chiou et al., 2002). A 3' *nor-1* integrant generates a 2.1 kb DNA fragment in the rapid PCR integration assay regardless of the size of the *nor-1* promoter in the reporter plasmid. The percentage of transformants that had a 2.1 kb band with the rapid assay varied from experiment to experiment ranging from 2 to 10% (data not shown). While the PCR assay could identify 3' integrants, it could not identify multiple integrants (multiple 3' integrations and/or additional non-3' integrations). Consequently, Southern hybridization analysis was used to confirm PCR integration assay results for each reporter construct. Disappearance of a 3.0 kb DNA fragment in the recipient strain, NR-1, and the appearance of 3.7 and 4.0 DNA fragments was diagnostic for 3' *nor-1* integration (Figure 4.5). Two confirmed 3' *nor-1* integrants for each *nor-1::GUS* reporter construct were used for solid culture and liquid culture GUS reporter analysis. *A. parasiticus* GUS reporter assays

The *nor-1::GUS* transformants were tested for GUS activity using liquid culture (Figure 4.6) and solid culture assays (Figure 4.7). Liquid culture measurement of GUS reporter activity was reported as the average GUS activity in 2 independent transformants per *nor-1::GUS* reporter construct analyzed in duplicate after growth for 48 h in GMS liquid shake cultures. The solid culture GUS activity assay was performed on 48 h colonies grown in duplicate on YES solid medium.

In order to determine if AfIR1 was necessary for *nor-1* transcriptional activation, fungal isolates carrying the 332, 332AfIRmut, 76 and 64 bp promoter fragments in *nor-1::GUS* constructs were tested for GUS activity using liquid culture (Figure 4.6) and solid culture (Figure 4.7A) activity assays. *A. parasiticus* isolates carrying the



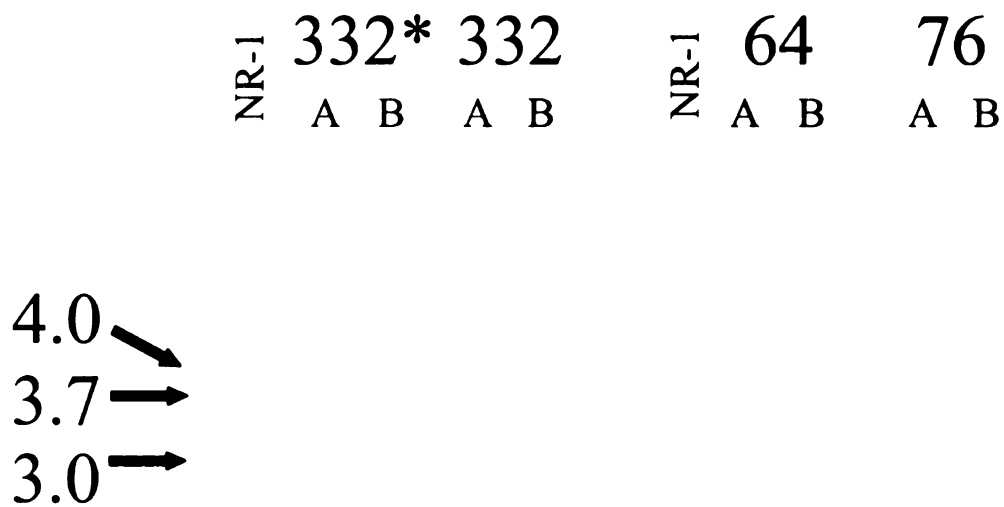


Figure 4.5. Southern hybridization analysis of *A. parasiticus* transformants with 332AflRmut (332*), 332, 76, 64 and NR-1. Each letter indicates a different isolate from the same *nor-1::GUS* construct. Two of the 3' integrants shown from each *nor-1::GUS* reporter construct were used for solid culture and liquid culture GUS assays.



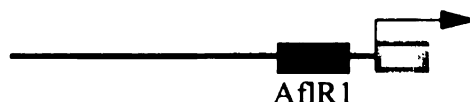
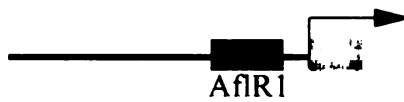



Sample		GUS Activity (pmol/min/mg)
3000		168 ± 115
1250		429 ± 442
1200		17.2 ± 8.1
664		16.0 ± 4.0
332P		21.1 ± 14.5
332		5.4 ± 2.2
332AflRmut		ND
NR-1		ND

Figure 4.6. Liquid culture GUS assay analysis was performed on two different 3' integrants from each *nor-1::GUS* reporter construct grown in duplicate. The GUS activity is reported as the mean pmol/min/mg ± the standard deviation.

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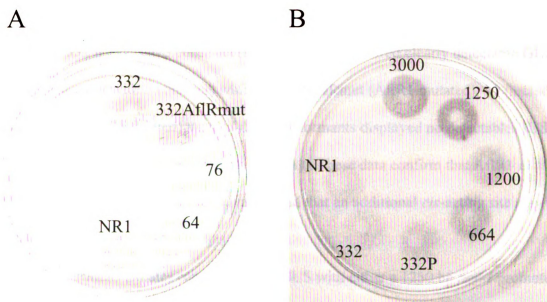


Figure 4.7. Solid culture GUS assay analyses were performed on different 3' integrants from each *nor-I::GUS* reporter construct on 46 h YES agar colonies. The recipient strain NR-1 was added as a negative control and had no detectable activity. (A) The 332 *nor-I::GUS* transformant (includes AflR1) had detectable GUS activity while the 332AflRMUT (AflR1 mutated), 76 (includes AflR1) and 64 (AflR1 deleted) *nor-I::GUS* transformants had no detectable GUS activity after 24 h incubation with GUS substrate. (B) The 3000 (includes AflR1, AflR2 and AflR3) and 1250 (includes AflR1 and AflR2) *nor-I::GUS* transformants had similar activity. The 1200, 664 and 332P (all 3 include AflR1 only) *nor-I::GUS* transformants all had similar activity which was significantly less than the 3000 and 1250 *nor-I::GUS* transformants. The 332 *nor-I::GUS* transformant (includes AflR1) had significantly less GUS activity than all other *nor-I::GUS* transformants shown.

332 *nor-1::GUS* construct (includes AflR1) converted 5.4 pmol/min/mg while the isolates carrying the 332AflRmut *nor-1::GUS* transformant (AflR1 mutated) had no detectable GUS activity (Figure 4.6). In agreement with these liquid culture data, isolates carrying the 332 *nor-1::GUS* construct (includes AflR1) showed clearly detectable GUS activity (blue color within colonies) while the 332AflRmut (AflR1 mutated), 76 (includes AflR1) and 64 (AflR1 deleted) *nor-1::GUS* transformants displayed no detectable GUS activity with the solid culture assay (Figure 4.7A). These data confirm that AflR1 is necessary for *nor-1* transcriptional activation and that an additional *cis*-acting site may be located between -76 and -332.

A. parasiticus isolates carrying *nor-1::GUS* with either a 1250 bp *nor-1* promoter fragment (includes AflR1 and AflR2) or a 1200 bp promoter fragment (includes AflR1 only) were analyzed to determine if AflR2 is necessary for *nor-1* transcriptional activation. Deletion of AflR2 resulted in at least a 10 fold reduction in liquid culture GUS activity (Figure 4.6). Fungal isolates carrying the 1250 bp promoter in the *nor-1::GUS* construct clearly demonstrated more activity than the 1200 bp promoter in the solid culture GUS assay as well (Figure 4.7B). These data strongly suggest that the presence of AflR2 in the *nor-1::GUS* construct results in greater *nor-1* expression.

The role of AflR3 in *nor-1* transcriptional activation was analyzed using two *nor-1::GUS* fusion constructs; one carrying a 3000 bp *nor-1* promoter fragment and the other a 1250 bp promoter fragment (Figure 4.6 and 4.7B). Fungal isolates carrying *nor-1::GUS* constructs with the 3000 bp fragment (includes AflR1, AflR2 and AflR3) and the 1250 bp fragment (includes AflR1 and AflR2) driving expression of *nor-1::GUS* had similar activities in both the liquid culture and solid culture GUS assays suggesting that AflR3 is not involved in *nor-1* transcriptional activation and that no additional *cis*-acting

sites for *nor-1* transcriptional activation are located between -1250 and -3000.

Fungal isolates carrying *nor-1::GUS* constructs with 1200, 664, 332P and 332 promoter fragments were analyzed to determine if there were any *cis*-acting sites located between -1200 and -332 in the *nor-1* promoter. Isolates carrying *nor-1::GUS* constructs with the 1200 and 664 promoter fragments (both include AflR1 only) had similar liquid culture (Figure 4.6) and solid culture (Figure 4.7B) activities which were greater than activity measured for isolates carrying the 332 promoter fragment. We hypothesized that the increased activity in these larger (1200 and 664 bp) promoter fragments was due either to the presence of a *cis*-acting site located between -332 and -664 or possibly due to negative effects of plasmid-derived sequences located adjacent to and upstream from the 332 bp promoter fragment. To distinguish between these possibilities, fungal isolates carrying *nor-1::GUS* with 332 and 332P promoter fragments were analyzed. The 332P promoter fragment has a 503 bp PCR product that includes the *pyrG* translational stop codon and transcriptional terminator inserted into pBNG332 between the *niaD* selectable marker and *nor-1* promoter at the *PacI* site (Figure 4.8). Fungal isolates carrying the 332P, *nor-1::GUS* construct (has AflR1 and *pyrG* 3') displayed a clear increase in GUS activities compared to isolates carrying the 332, *nor-1::GUS* construct in both the liquid culture (Figure 4.6) and solid culture GUS assays (Figure 4.7B). In addition, isolates carrying the 332P, *nor-1::GUS* construct had similar GUS activity to the isolates carrying the 664 and 1200 *nor-1::GUS* constructs as measured in both liquid culture (Figure 4.6) and solid culture (Figure 4.7B) assays indicating that the difference in activity between 332 and 664 is likely not due to a specific *cis*-acting site in that region.

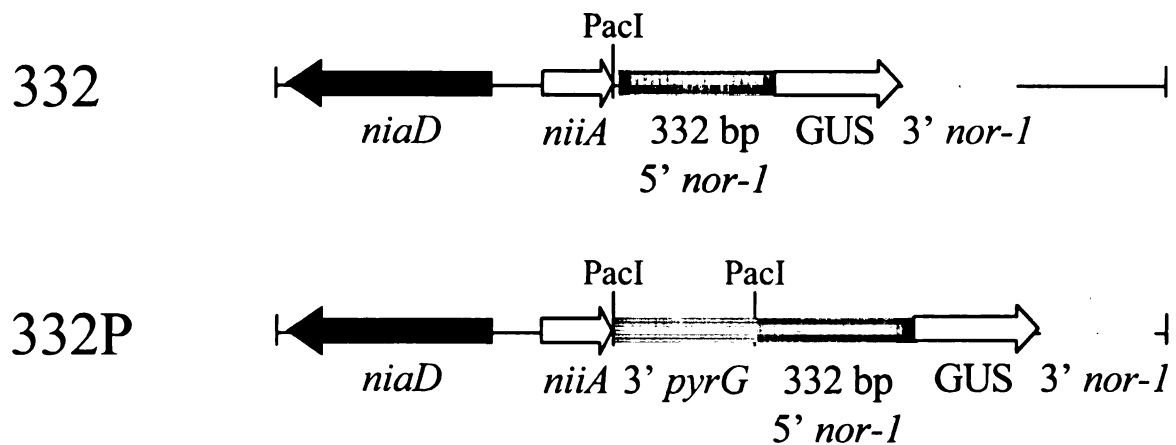


Figure 4.8. Genetic map of 3' integrants for both 332 and 332P *nor-1::GUS* transformants. 3' integration with the plasmids used in the *A. parasiticus* study result in the *niaD* selectable marker being immediately upstream of the *nor-1::GUS* promoter. Included in the 7.4 kb *niaD* fragment is 680 bp of *niiA* coding sequence. A 503 bp PCR product that included the *pyrG* translational stop codon and transcriptional terminator was inserted into pBNG332 between the *niaD* selectable marker and *nor-1* promoter at the *PacI* site.



DISCUSSION

Since its discovery in *A. flavus* in 1993 (Payne et al., 1993), AflR frequently has been described as “the” aflatoxin biosynthesis pathway regulator. Experiments with AflR null mutants and AflR inducible expression strains along with AflR-DNA binding studies have, at least in part, supported this designation (reviewed in introduction). However, electrophoretic mobility shift assays with *A. parasiticus* recombinant AflR and fungal protein extracts suggested that *A. parasiticus* AflR either does not bind AflR1 in the *nor-1* promoter or has a much lower affinity for AflR1 compared to consensus AflR binding sites in several other aflatoxin biosynthetic promoters (Ehrlich et al., 1999b) casting doubt on the functional significance of AflR1. In contrast to these observations, we demonstrated that recombinant *A. flavus* tAflR binds to AflR1 *in vitro*. Because the consensus AflR1 sequence is identical in *A. parasiticus* and *A. flavus* and because *A. parasiticus* AflR complements a AflR null mutant in *A. flavus* (Chang et al., 1993), we hypothesize that native AflR binds to the AflR1 site in the *nor-1* promoter in both species *in vivo*. We tested this hypothesis by analyzing the effects of deletion and replacement mutations on *nor-1* promoter function using *nor-1::GUS* reporter constructs.

In previous studies, three aflatoxin biosynthetic gene promoters have been studied in some detail: *avnA* with two consensus AflR binding sites (*A. parasiticus*; Cary et al., 2000), *pksA* (*A. parasiticus*; Ehrlich et al., 2002) and *stcU* (*A. nidulans*; Fernandes et al., 1998) with three consensus AflR binding sites each. With *avnA*, only one of the AflR binding sites was functional. The most distal AflR binding site in *pksA* (AflR1) and *stcU* had no impact on transcriptional activation of their respective genes. The other two AflR binding sites in the *pksA* promoter, AflR2 and AflR3, are both needed for maximal *pksA*



transcription. With *stcU*, only one of the two functional AfIR binding sites is necessary for maximal *stcU* transcription. In our study, deletion of AfIR1 in the *A. parasiticus* or *A. flavus nor-1* promoter greatly decreased GUS activity. In addition, substitution of only nucleotide residues in AfIR1 in the 332 base pair *A. parasiticus nor-1* promoter also resulted in a significant decrease in GUS activity. These data confirm that AfIR1 is necessary for *nor-1* transcriptional activation in *A. flavus* and *A. parasiticus*. It is currently unknown if the difference in GUS activity between the 138 and 103 *nor-1::GUS* transformants in *A. flavus* is biologically significant.

Comparison of GUS activity data between *A. parasiticus* isolates carrying *nor-1::GUS* with the 1250 and 3000 bp promoter fragments suggests that there are no key *cis*-acting sites located in that region that are necessary for *nor-1* transcriptional activation supporting the conclusion that AfIR3 is non-functional with respect to *nor-1* expression. Others have shown that AfIR3 is clearly required for *pksA* expression (Ehrlich et al., 2002). Perhaps the most surprising observation in our study was that deletion of 50 nucleotide residues containing the AfIR2 site in the 1250 *nor-1::GUS* promoter fragment resulted in at least a 10 fold reduction in GUS activity; deletion of AfIR1 then reduced GUS activity to non-detectable levels. These data allow us to conclude that AfIR1 is necessary for full *nor-1* expression (332 bp *nor-1::GUS* activity defined as full activity). The data also strongly suggest that AfIR2 is necessary for greater *nor-1* expression.

We propose two alternative models to explain these data: 1) AfIR2 works synergistically with AfIR1 to mediate transcription of the *nor-1* promoter; or 2) AfIR2 mediates expression of ORF3 (potentially encodes a polypeptide of approximately 300 amino acid residues - Figure 4.4) directly downstream from AfIR2 which directly or indirectly impacts transcription of the *nor-1* promoter. Identification of a cDNA

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corresponding to ORF3 in an *A. parasiticus* cDNA library (a generous gift from Dr. Jeff Cary, USDA) strongly suggests it represents a functional gene. Interestingly, blast searches using ORF3 as a query sequence have not provided solid clues regarding potential function. Model 2 allows us to make 2 related predictions regarding *nor-1* promoter function. 1) Since fungal isolates carrying *nor-1::GUS* constructs with the 3000 and 1250 bp promoter fragments carry two copies of ORF3 with AflR2 and show the highest GUS expression levels, accumulation of additional ORF3 protein in strains with two copies overcomes a protein threshold resulting in extreme upregulation of *nor-1* promoter activity. 2) Loss of ORF3 function due to AflR2 deletion in the 2nd copy accounts for downregulation (or lack of upregulation) of both *nor-1* and *pksA* expression. Similar results are seen with the insertion of an additional copy of AflR (Chang et al., 1995) suggesting a possible regulatory role for ORF3. Transcriptional read-through from ORF3 may also explain the affect seen with AflR2. These predictions will be analyzed in follow-up studies.

While it is clear that AflR is a key positive regulator of aflatoxin biosynthesis, the case for AflR being the sole regulator of all aflatoxin biosynthesis structural genes is not as strong. For example, AflJ has been putatively assigned a role as a transcriptional co-activator (Chang et al., 2001) and has been reported to directly interact with AflR (Chang and Yu, 2002). It is unknown how AflJ acts as a transcriptional co-activator. Studies with the *pksA* promoter (Ehrlich et al., 2002) found evidence that both PacC (pH sensing) and BrlA (sporulation) can impact *pksA* transcriptional regulation through *cis*-acting sites in the *pksA/nor-1* intergenic region (see Figure4.4). However, deletion of these consensus *cis*-acting sites for PacC and BrlA do not affect *nor-1* transcriptional regulation in *A. parasiticus* under the culture conditions tested here.

Solid culture GUS analysis of fungal isolates carrying *nor-1::GUS* constructs with the 332 and 76 bp promoter fragments indicate that an additional *cis*-acting site(s) located between -332 and -76 contributes to *nor-1* transcriptional activation. Because no additional consensus AflR binding sites (TCGnnnnnCGR) can be found in this promoter region, it follows that one or more unknown transcriptional activators(s) bind to this promoter region and influence *nor-1* transcriptional activation in *A. parasiticus*. In support of this idea, recent experiments have localized 3 additional *cis*-acting sites located in the 332 bp *nor-1* promoter fragment (Miller and Linz, unpublished data) which influence *nor-1* promoter function *in vivo*. In addition, protein extracts have been shown to specifically bind to these *cis*-acting sites using electrophoretic mobility shift assays (Miller and Linz, unpublished data). However, we can not rule out the possibility that AflR binds to additional non-consensus AflR binding sites in the *nor-1* promoter. Our studies with *A. flavus* demonstrate that AflR1 is necessary for *nor-1* transcriptional activation but evidence is lacking for additional transcription factor(s) binding sites.

The regulation of middle and late aflatoxin biosynthetic genes is also possibly regulated by additional transcriptional factors. With a full length *stcU* promoter fused to GUS, only a 2-3 fold increase in activity was found compared to an AflR mutant strain (Fernandes et al., 1998). In addition, substitution of both AflR binding sites only resulted in an approximately 5 fold reduction (not reduced to baseline expression levels) in GUS activity (Fernandes et al., 1998). Due to the relatively high activity in the AflR mutant strain and the moderate decrease resulting from binding site substitution, the possibility exists that other transcriptional activators besides AflR are involved in *stcU* transcriptional activation (Fernandes et al., 1998). For the middle gene *avnA* in

A. parasiticus, substitution of the AfIR binding site resulted in a 10 fold decrease in promoter activity (Cary et al., 2000).

A possible shortcoming of many aflatoxin promoter studies is that the integration site for reporter constructs was located outside of the aflatoxin gene cluster (eg *trpC* for *stcU::GUS* and *niaD* for *avnA::GUS*) (Fernandes et al., 1998 and Cary et al., 2000).

Previous studies have revealed a greater than 500 fold decrease in promoter activity with integration of *ver-1::GUS* at *niaD* versus integration at *ver-1* (Liang et al., 1997). While it appeared that the timing of transcription was the same, the magnitude was severely affected (Liang et al., 1997). A similar effect has also been reported at the *pyrG* locus (Chiou et al., 2002). In our current work, the *A. parasiticus nor-1::GUS* reporter constructs were integrated at the *nor-1* locus at which we have demonstrated “normal” timing and level of transcription as compared to the wild type *nor-1* gene (Chiou et al., 2002).

While it is known that insertion of aflatoxin biosynthetic genes at *niaD* results in a dramatic down regulation of aflatoxin gene transcription, it is unknown if insertion of *niaD* into the aflatoxin gene cluster can have an effect on neighboring aflatoxin gene transcription. 3' integration with the plasmids used in the *A. parasiticus* study result in the *niaD* selectable marker being immediately upstream of the *nor-1::GUS* promoter (Figure 4.8). Included in the 7.4 kb *niaD* fragment in pNANG is 680 bp of *niiA* coding sequence. A 503 bp PCR product that includes the *pyrG* translational stop codon and transcriptional terminator was inserted into pBNG332 between the *niaD* selectable marker and *nor-1* promoter at the *PacI* site (Figure 4.8). Fungal isolates carrying the 332P, *nor-1::GUS* construct (has AfIR1 and *pyrG* 3') had significantly higher GUS activity than isolates carrying the 332, *nor-1::GUS* construct (Figure 4.6B and 4.7). In

addition, isolates carrying the 332P, *nor-1*::GUS construct had similar GUS activity to the 664 and 1200 *nor-1*::GUS transformants (Figure 4.6B and 4.7) indicating that the difference in activity between 332 and 664 may not be due to a specific *cis*-acting site in that region of the *nor-1* promoter. Rather, the neighboring *niaD/niiA* genes appear to have a negative affect on *nor-1* transcriptional activation that can be mitigated by inserting the *pyrG* fragment (or native sequences) as a spacer.

In summary, we demonstrated recombinant *A. flavus* AflR binding to an oligonucleotide containing the proposed AflR binding site AflR1 using the Southwestern blot procedure. Additionally, deletion analysis with *nor-1*::GUS reporter strains showed that AflR1 is necessary for *nor-1* transcriptional activation in *A. flavus*. Using *nor-1*::GUS reporter strains containing *nor-1* promoter deletions and substitutions, we demonstrated that in *A. parasiticus*, AflR1 and possibly AflR2 and additional *cis*-acting site(s) are necessary for *nor-1* transcriptional activation. We also tentatively identified a novel gene (ORF3) that may function to directly or indirectly activate *nor-1* transcription in *A. parasiticus*.

ACKNOWLEDGMENTS

Chapter 4 has been submitted for publication. (Miller MJ, Brown-Jenco C, OBrian G, Payne GA and Linz JE. submitted 2003. Role of AflR in *nor-1* transcriptional activation in *Aspergillus flavus* and *A. parasiticus*. Applied and Environmental Microbiology.) All experiments with *A. parasiticus* described in Chapter 4 were performed by Michael Miller. Michael Miller also was the primary author.

CHAPTER 5

Identification of novel *cis*-acting sites in the aflatoxin biosynthetic *nor-1* promoter of *Aspergillus parasiticus*

INTRODUCTION

Aflatoxin is a potent hepatocarcinogen that can contaminate several commodities including corn, cotton, peanuts and certain tree nuts (Council for Agricultural Science and Technology, 2003). Of the four species of *Aspergillus* that make aflatoxin, only two are of economic importance, *A. flavus* and *A. parasiticus* (Council for Agricultural Science and Technology, 2003). In areas of the world with high aflatoxin exposure, aflatoxin and hepatitis B interact as risk factors for human liver cancer (Scholl et al., 1995). Due to health concerns, several countries have established limits for aflatoxin contamination including the United States. As a result, more than \$100 million is estimated to be lost in the United States each year due to aflatoxin contamination (Robens, 2001). Due to health and economic concerns, it is desirable to reduce or eliminate aflatoxin from the food chain.

A potential means to accomplish this goal is to elucidate the molecular mechanisms that regulate aflatoxin biosynthesis. This information is likely to generate novel approaches and targets for inhibition of aflatoxin gene expression. Aflatoxin biosynthesis is a complex process that requires at least 16 different enzymatic steps (Bhatnagar et al., 1994). All identified aflatoxin structural genes reside in a 70 kb gene cluster and appear to be co-regulated (Trail et al., 1995b). In addition to several

structural genes, the aflatoxin gene cluster also contains at least one regulatory gene, *afIR* (Payne et al., 1993). Several pieces of evidence demonstrate a key regulatory role for AfIR in aflatoxin biosynthesis (reviewed in Miller et al., 2003a). Although it is clear that AfIR is a key regulator of aflatoxin synthesis, it is not clear if AfIR is the only specific transcription factor needed for transcriptional activation of all aflatoxin biosynthesis genes.

To address this question, we have focused attention on expression of the *nor-1* gene (Chiou et al., 2002, Miller et al., 2003a, Miller et al., 2003b). Nor-1 catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin (Trail et al., 1994 and Zhou and Linz, 1999). Previously identified *cis*-acting sites in the *nor-1* promoter include a consensus AfIR binding site (TCGnnnnnCGR) located at -75 to -64 bp (AfIR1) from the primary *nor-1* transcriptional start site (+1); additional upstream consensus AfIR binding sites are located at -1213 (AfIR2) and -1563 (AfIR3). Genes upstream from *nor-1* include an open reading frame of unknown function (ORF3: translational start at -1073) and the divergently transcribed *pksA* (translational start at -1731) (Ehrlich et al., 2002 and Miller et al., 2003a).

Initial experiments with a 3.0 kb *nor-1* promoter fused to the GUS (*uidA*) reporter gene validated the use of the GUS reporter system (Chiou et al., 2002). In addition, valuable screening techniques were developed including a rapid PCR site of integration assay and a solid culture GUS assay (Chiou et al., 2002). Subsequent analysis using these *nor-1* reporter constructs demonstrated that AfIR1 and possibly AfIR2 are necessary for maximum *nor-1* transcriptional activation *in vivo* under aflatoxin inducing conditions (Miller et al., 2003a). Preliminary experiments also identified a potential *cis*-acting site located between -76 and -332 in the *nor-1* promoter (Miller et al., 2003a).

Only 5 *A. parasiticus* aflatoxin biosynthetic genes have had their transcriptional start point experimentally determined: *nor-1* (Trail et al., 1994), *avnA* (Cary et al., 2000), *pksA* (Ehrlich et al., 2002), *ver-1* (Skory et al., 1992) and *aflR* (Ehrlich et al., 1999a). While putative TATA boxes have been identified in 4 of them (all but *aflR*), only the TATA box in *avnA* has been functionally analyzed (Cary et al., 2000). Deletion of the TATA box in the *avnA* promoter resulted in at least a 5 fold reduction in *avnA* transcriptional activation (Cary et al., 2000). Unfortunately, deletion of the TATA box also resulted in deletion of a functional *AflR cis*-acting site and perhaps other functional *cis*-acting sites. Replacement of the TATA box in the context of a wild type promoter would provide direct evidence for TATA box function in any promoter of interest including *avnA*.

The objective of the current study was to further define *cis*-acting sites that are necessary for maximum *nor-1* transcriptional activation in *Aspergillus parasiticus* under aflatoxin inducing conditions. Specifically, we wished to: 1) test the *in vivo* function of a putative TATA box in the *nor-1* promoter, 2) further refine the location of a *cis*-acting site previously shown to occur between nucleotide residues -76 and -332 (+1 is transcriptional start) using *nor-1* reporter constructs *in vivo*, and 3) test for *in vitro* protein binding to potential *cis*-acting sites. Although putative TATA boxes have been identified in aflatoxin biosynthetic promoters, this was the first direct functional analysis of a TATA box in an aflatoxin gene promoter. Substitution of the TATA box in the context of a larger *nor-1* promoter resulted in non-detectable GUS activity. We identified a novel *cis*-acting site (norL) located between -210 and -238 that is necessary for maximum *nor-1* transcriptional activation *in vivo*. Using electrophoretic mobility shift assays, we demonstrated specific protein binding to norL (NorLbp) and an

additional site, CRE1 (CREbp). Both norLbp and CRE1bp appear to rely on functional AfIR for maximum DNA binding. Lastly, we propose a model for how AfIR, NorLbp and CREbp may interact to govern *nor-1* transcriptional activation.

MATERIALS AND METHODS

Strains and growth conditions

Escherichia coli DH5 α F'c [F' *endA1 hsdR17* (*r_k*- *m_k*-) *supE44 thi-1 recA gyrA* (NaI') Δ *relA1* (*lacZYA argF*)_{u169}:(m80 Δ *lacZ* M15)] (Invitrogen, Carlsbad, CA) was used to amplify plasmid DNA using standard procedures (Ausubel et al., 2003). *Aspergillus parasiticus* NR1 (*niaD*) was used as the recipient strain for all fungal transformations (Hornig et al., 1990). *A. parasiticus* SU1 is a wild type strain. *A. parasiticus* AFS10 is an *aflR1* knock-out strain (Cary et al., 2002).

To measure solid culture GUS activity, YES agar (2% yeast extract, 6% sucrose; pH=5.8) plates were used. To measure liquid culture GUS activity, GMS (Buchanan and Lewis, 1984) liquid media was used as previously described (Miller et al., 2003a). To extract protein for electrophoretic mobility shift assays, GMS (Buchanan and Lewis, 1984) liquid media was used.

Plasmid Constructs

(i) pNANG-3. The construction of pNANG-3 was previously described (Miller et al., 2003a). pNANG-3 contains the *niaD* selectable marker, GUS (*uidA*) reporter enzyme and *nor-1* terminator. Appropriate promoter pieces, amplified by PCR using primers with *NotI* and *PacI* tails, can be directionally cloned into pNANG-3 resulting in pBNG-n

(where n designates the size of the promoter in base pairs). pAPGUSNN-B (Chiou et al., 2002) was used as a template for PCR amplification of all *nor-1* promoter fragments. pBNG-n will have the PCR amplified promoter driving the transcription of the GUS reporter gene.

(ii) pBNG332 and pBNG332TATAmut. The polyadenylation site for ORF3 (Miller et al., 2000a) is 332 bp upstream from the transcriptional start site of *nor-1*. A 332 bp *nor-1* promoter piece was amplified by PCR with appropriate primers with *PacI* (JL267: 5'-GTTAATTAAGTCG AGCGGACATGGCCACG-3') and *NotI* tails (JL186: 5'-TCGCGGCCGCTAAGTGATCCATTC ATTATGTC-3'). For pBNG332TATAmut, the TATA box was mutated to a *PmeI* site (5'-ATATATAG-3' to 5'-GTTTAAAC-3') in the context of the 332 bp *nor-1* promoter. The 332 bp *nor-1* promoter was divided into two PCR fragments (Pac/Pme and Not/Pme) that joined at the TATA box. The primers used for the Pac/Pme fragment were JL267 and JL414 (5'-ATGTTTAA ACTGGGATAC GATCATGGGTC-3'). The primers used for the Not/Pme fragment were JL186 and JL415 (5'-GGGTTTAAACGGCGGTG TGTTGGTCG-3'). After digestion with the appropriate restriction endonucleases, a three piece ligation was performed with pNANG-3, Pac/Pme fragment and Not/Pme fragments. The sequence of the *nor-1* promoter in both pBNG332 and pBNG332TATAmut was verified by sequence analysis.

(iii) pBNG332, pBNG298, pBNG268, pBNG238 and pBNG210. To generate the *nor-1* promoter deletion series, different upstream PCR primers with *PacI* tails were used with the same downstream PCR primer (JL186) that contained a *NotI* tail. The upstream primers used were: JL267 for pBNG332, JL411 (5'-CCTTAATTAAACTGCTATGGTG ACCTATTG-3') for pBNG298, JL412 (5'-CATTAATTAACCACATAGGCTACTCAA AAT-3') for pBNG268, JL413 (5'-GGTTAATTAAAGATCTCTGCTATTAAGTCGG-3')

for pBNG238 and JL302 (5'-C CCTTAATTAATAGCGTGCTGGATGCGCGAA-3') for pBNG210.

(iv) pBNGnorLmut. For pBNGnorLmut, the -210 to -238 region was substituted (5'-AG ATCTCTGCTATTAAGTCGGTGATTAG-3' to 5'-GTATAAGAAGTTTGTGATGGGATTCGT C-3') in the context of the 332 bp *nor-1* promoter. The 332 bp *nor-1* promoter was divided into two PCR fragments (Pac/210 and Not/238) that joined at -224. The primers used for the Pac/210 fragment were JL267 and JL613 (5'-CAAACCTTCTTATACGCTCATGTCAATTTTGAG-3'). The primers used for the Not/238 fragment were JL186 and JL 612 (5'-TGATGGGATTCGTCCGTGCTGGATGCGC-3'). JL612 and JL613 do not include a restriction endonuclease tail. After digestion with the appropriate restriction endonucleases, a three piece ligation was performed with pNANG-3, Pac/210 fragment and Not/238 fragment. The sequence of the *nor-1* promoter in pBNG332norLmut was verified by sequence analysis.

Generation and Screening of Transformants

Transformation

Transformation of *A. parasiticus* protoplasts was performed as described by Horng et al. (1990). 2-4 µg of DNA was used with 10^7 protoplasts resulting in approximately 100 transformants.

Site of Integration PCR Assay

A rapid DNA extraction procedure and PCR analysis were performed as described by Chiou (Chiou et al., 2002). Following PCR, a 2.0 kb DNA fragment is diagnostic for 3' integration.

Southern Hybridization

Genomic DNA was purified from *A. parasiticus* cultures shaken for 48 h in 100 ml YES (2% yeast extract, 6% sucrose and pH=5.8) liquid medium at 29°C with five, 6 mm glass beads (Skory et al., 1993) and subjected to standard agarose gel electrophoresis (Ausubel et al., 2003). Southern hybridization analysis was performed according to standard procedures (Ausubel et al., 2003). 2.5 µg of genomic DNA was digested with *ScaI* and probed with a 900 bp *Clal* fragment isolated from the *nor-1* terminator region of pAPGUSNN-B (Chiou et al., 2002). Digestion of DNA from the recipient strain NR-1 generates a 3.0 kb restriction fragment while a 3' integrant results in 3.7 and 4.0 kb restriction fragments.

GUS Reporter Assays

Solid culture GUS Assay

Autoclaved Nytran SPC (Schleicher & Schuell, Keene NH) membranes were first placed on top of YES (2% yeast extract, 6% sucrose, 1.5% agar and pH=5.8) agar plates. Transformants were then transferred via sterile toothpick to the Nytran membrane. After incubation for 46 h at 29°C in the dark, the Nytran filters were removed, frozen in liquid nitrogen and thawed at room temperature (2 repetitions) and then incubated for up to 24 h with GUS substrate solution that includes 0.04% of the colorimetric GUS substrate X-Glu (5-bromo-4-chloro-3-indolyl β-D-glucuronide) in GUS reaction buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 0.07% β-mercaptoethanol) and visually evaluated.

Liquid Culture GUS Assay

Two confirmed *A. parasiticus* 3' transformants containing reporter constructs were grown in duplicate for 48 h at 29°C in the dark with shaking in 100 ml of GMS (Buchanan and Lewis, 1984) liquid medium in a 250 ml flask with five 6 mm glass beads. Mycelia were collected by filtration through Miracloth (Calbiochem, La Jolla CA) and ground in a mortar with a pestle under liquid N₂. Approximately 200 mg of ground mycelia was transferred to a 1.5 mL microcentrifuge tube and kept on ice. After all the samples had been collected, 500 mL of GUS lysis buffer (50 mM NaH₂PO₄ [pH=7], 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 0.07% β-mercaptoethanol and 25 mg/ml PMSF [phenylmethylsulfonyl fluoride]) was added. The samples were vortexed for 15 s and then centrifuged for 10 min at 10,000 X g at 4°C. The supernatant was withdrawn and placed in a new tube. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules CA). GUS activity was determined with 100 µg of each sample incubated at 30°C with 145 µg of the GUS substrate 4-methylumbelliferyl β-D-glucoside (MUG) in a final volume of 200 µl. At 0, 10 and 30 min, 800 µl of stop solution (200 mM Na₂CO₃) was added. After stopping the GUS reaction, 200 µl of sample was loaded into a microtiter plate which was analyzed using a Cytofluor II fluorometer (Biosearch Co., Bedford MA). Excitation and emission wavelengths were 360 nm and 460 nm, respectively. To evaluate the fluorometer results, a standard curve of 4-methylumbelliferone (10 nM to 600 nM) in GUS lysis buffer was also read in the fluorometer. GUS activity was reported as pmols 4-methylumbelliferone / minute / mg protein.

in vitro DNA Binding Assays

Protein Extraction

Cellular protein was extracted from cultures of *A. parasiticus* SU1 using modifications of the methods of Peters and Perez-Esteban (Peters and Caddick, 1994 and Perez-Esteban et al., 1993). 1 liter flasks containing 10 6mm glass beads and 500 mL medium (GMS₁₀ or PMS₁₀) was inoculated with 1 X 10⁸ spores. The cultures were incubated for 48 hours at 29°C in the dark with shaking at 150 rpm. The mycelia was filtered through miracloth (Calbiochem, La Jolla CA), washed with cold, sterile water, frozen with liquid nitrogen and stored at -80°C. Frozen mycelia was ground using mortar and pestle with liquid nitrogen and transferred to a tared 125 ml flask. 5 ml of lysis buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT and 1 mM PMSF) per gram of ground mycelia was added to the flask. In addition, 1 ml of protease inhibitor cocktail (Sigma, St. Louis MO - product # P8215) was added to the flask per gram of mycelia. After stirring on ice for 15 minutes, saturated ammonium sulfate was slowly added to a final concentration of 10%. The suspension was then stirred on ice for 15 minutes and then set idle for 15 minutes on ice. Cell debris was then pelleted at 100,000 x g (30 minute spin at 4°C) and the volume of the supernatant, determined using a graduated cylinder, was transferred to a 50 ml flask. Then solid ammonium sulfate was added slowly over 1.5 hours to raise the concentration from 10% to 70%. The ammonium sulfate addition was done while stirring on ice. After all ammonium sulfate was added, the flask was incubated for 30 minutes on ice without stirring. The protein was pelleted at 10,000 X g (20 minutes at 4°C). The pellet was resuspended in dialysis buffer (15% glycerol, 15 mM Hepes-KOH (pH 7.9), 100 mM KCl, 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail (1 ml per

20 grams of mycelia) and dialyzed 2X in 2 liters dialysis buffer using a 10K slide-a-lyzer (Pierce, Rockford IL). The protein concentration was determined using the BioRad (BioRad, Hercules CA) protein dye reagent following manufacturers instructions. The dialyzed solution was then aliquoted and stored at -80°C.

Probe Generation

Probes were end-labeled with $\gamma^{32}\text{P}$ using Ready-to-Go Kinase following the manufacturers instructions (Amersham, Piscataway NJ). The probes and competitors used are listed in table 5.1.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed essentially as described in Current Protocols in Molecular Biology (Ausebel et al., 2003). Five percent acrylamide (80:1 acrylamide:bis-acrylamide) non-denaturing gels were used. 20 fmol of *nor-1* and *ver-1* promoter probes were incubated for 15 minutes at 30°C with 2 μg dIdC, 7.5 μg BSA and competitor (if desired) with 32 μg protein extract (added last). The entire binding reaction volume was 25 μl which consisted of 20 μl of dialysis buffer in order to keep the glycerol concentration of the binding reaction greater than 10%.

RESULTS

Generation and screening of *A. parasiticus* transformants

In order to identify *cis*-acting sites necessary for *nor-1* transcriptional activation in *A. parasiticus*, several *nor-1*::GUS reporter plasmids were constructed (Figure 5.1 and

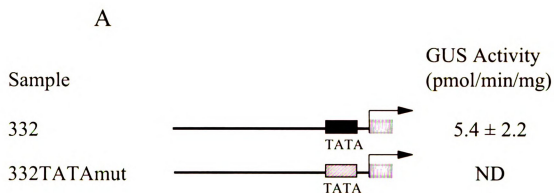
Table 5.1. Oligonucleotides used for electrophoretic mobility shift assays

Oligo	Location ^a	Sequence ^b
206/244	-244 to -205	ATG AGC AGA TCT CTG CTA TTA AGT CGG TGA TTA GCG TGC T
206/244 mut	-244 to -205	ATG AGC <u>GTA TAA GAA GTT TGT GAT GGG ATT</u> <u>CGT CCG</u> TGC T
CRE1	+4 to +31	TTC TAA GCC GTG ACA TAA TGA ACG GAT C
CRE1mut	+4 to +31	TTC TAA GCC GTG <u>TGA</u> TAA TGA ACG GAT C
CRE2	-258 to -231	TAC TCA AAA TTG ACA TGA GCA GAT CTC T
CRE2mut	-258 to -231	TAC TCA AAA TTG <u>TGA</u> TGA GCA GAT CTC T

^a Location in relationship to the *nor-1* transcriptional start site. ^b Mutated bases are underlined, ATG is italicized in CRE1/CRE1mut and the possible *cis*-acting sites are bold in CRE1 and CRE2.

5.2). We defined the upstream border of the *nor-1* promoter as the polyadenylation site for ORF3 (Miller et al., 2003a), located 332 bp upstream from the primary transcriptional start site of *nor-1*. Transformants carrying a substitution of the putative TATA box (5'-ATATATAG-3' to 5'-GTTTAAAC-3') in the context of the 332 bp *nor-1* promoter were used to test TATA box function in the *nor-1* promoter. Deletion analysis generated clones with 332, 298, 268, 238 and 210 bp *nor-1* promoter fragments carried on *nor-1::GUS* reporter constructs. GUS activity in transformants that carried these plasmids was analyzed to localize a candidate *cis*-acting site. In addition, transformants carrying a replacement of the *norL* region (-210 to -238) were also analyzed.

Each *nor-1::GUS* reporter construct could theoretically integrate into the *A. parasiticus* chromosome by homologous recombination at three independent sites: *niaD*,



B

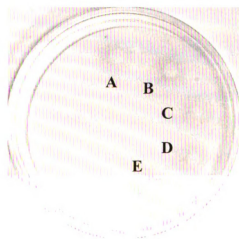
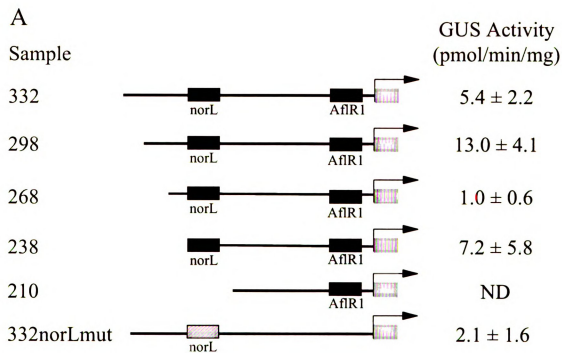


Figure 5.1. Importance of the TATA box in the *nor-1* promoter. (A) A liquid culture GUS assay analysis was performed on two different 3' integrants from each *nor-1*::GUS reporter construct (332 and 332TATAmut) grown in duplicate. GUS activity is reported as the average of 4 values in pmol/min/mg \pm the standard deviation. (B) Solid culture GUS assays were performed on different 3' integrants from each *nor-1*::GUS reporter construct on 46 h YES agar colonies. Colonies analyzed are: A 332-1, B 332-2, C 332TATAmut-1, D 332TATAmut-2, and E NR1.

Figure 5.2. Identification of *norL* cis-acting site. (A) A liquid culture GUS assay was performed on two different 3' integrants from each *nor-1::GUS* reporter construct grown in duplicate. The GUS activity is reported as the average of 4 values in pmol/min/mg \pm the standard deviation. (B) Solid culture GUS assays were performed on different 3' integrants from each *nor-1::GUS* reporter construct on 46 h YES agar colonies. Colonies analyzed are: A 332-1, B 298-1, C 268-1, D 238-1, E 210-1, G 332*norL*mut-1 and H NR1.



B



Figure 5.2

5' *nor-1* (*nor-1* promoter) and 3' *nor-1* (*nor-1* terminator). Screening for clones in which integration occurred at the 3' *nor-1* locus was essential for two reasons: 1) *niaD* integrants have been shown to have lower transcriptional activity than aflatoxin cluster integrants (Liang et al., 1997 and Chiou et al., 2002); and 2) 5' integrants result in the chromosomal *nor-1* promoter fused to the GUS gene and the plasmid *nor-1* promoter fused to the chromosomal *nor-1* gene. Transformants from the *nor-1::GUS* reporter constructs were screened initially by a rapid PCR assay specific for *nor-1*, 3' integration (Chiou et al., 2002). A 3' *nor-1* integrant generates a 2.1 kb DNA fragment in the rapid PCR integration assay regardless of the size of the *nor-1* promoter in the reporter plasmid. The percentage of transformants that had a 2.1 kb band with the rapid assay varied from experiment to experiment ranging from 2 to 5% (data not shown). While the PCR assay could identify 3' integrants, it could not identify multiple integrants (multiple 3' integrations and/or additional non-3' integrations). Consequently, Southern hybridization analysis was used to confirm PCR integration assay results for each reporter construct. Disappearance of a 3.0 kb DNA fragment in the recipient strain, NR1, and the appearance of 3.7 and 4.0 DNA fragments was diagnostic for 3' *nor-1* integration (data not shown). Two confirmed 3' *nor-1* integrants for each *nor-1::GUS* reporter construct were used for solid culture and liquid culture reporter analysis.

In order to determine if the TATA box was necessary for *nor-1* transcriptional activation *in vivo*, fungal isolates carrying the 332 and 332TATAmut promoter fragments in *nor-1::GUS* constructs were tested for GUS activity using liquid culture (Figure 5.1A) and solid culture (Figure 5.1B) activity assays. *A. parasiticus* isolates carrying the 332 *nor-1::GUS* construct (includes TATA) converted 5.4 pmol/min/mg while the isolates carrying the 332TATAmut *nor-1::GUS* transformant (TATA mutated) had no detectable

GUS activity (Figure 5.1A). In agreement with these liquid culture data, isolates carrying the 332 *nor-1*::GUS construct (includes TATA) showed clearly detectable GUS activity (blue color within colonies) while the 332TATAmut (TATA mutated) *nor-1*::GUS transformants displayed no detectable GUS activity. These data confirm that the TATA box is necessary for *nor-1* transcriptional activation in vivo.

Previously, a potential *cis*-acting site in the *nor-1* promoter was localized between -332 and -76 (Miller et al., 2003a). Isolates carrying *nor-1*::GUS constructs with the 210 and 76 promoter fragments had no detectable liquid culture or solid culture GUS activity (data not shown). Fungal isolates carrying *nor-1*::GUS constructs with 332, 298, 268, 238 and 210 promoter fragments were analyzed to localize the potential *cis*-acting site located between -332 and -210 in the *nor-1* promoter. Isolates carrying *nor-1*::GUS constructs with the 332, 298, 268 and 238 promoter fragments all had detectable liquid culture GUS activity (Figure 5.2A) and solid culture GUS activity whereas the 210 promoter fragment had reproducibly less activity (Figure 5.2B). To verify the *in vivo* significance of the 210 to 238 region (norL), isolates carrying a *nor-1*::GUS construct with norL replaced in the context of the 332 bp *nor-1* promoter fragment was used. Replacement of norL resulted a 2.5 fold reduction in liquid culture GUS activity (Figure 5.2A). In addition, replacement of norL also resulted in a decrease in solid culture GUS activity (Figure 5.2B). While a functional norL site is not sufficient for *nor-1* transcriptional activation, it is necessary for maximum *nor-1* transcriptional activation in the context of the 332 promoter.

in vitro analysis *nor-1* promoter elements

To test for protein affinity for specific DNA sites in the *nor-1* promoter,

electrophoretic mobility shift assays (EMSA) were used. The probes and competitors used for EMSA are described in Table 1. Total cellular protein used for EMSA was extracted from two different strains: SU1 (wild type) and AFS10 (*aflR* knock-out). Extracts from each strain were prepared twice on two separate occasions. Comparison of the shifted complexes generated with each extract provided evidence regarding the role of AflR in the activity.

The 206/244 oligo spans the *norL cis*-acting site that was identified using *nor-1::GUS* reporter strains *in vivo* (Figure 5.2) and was used as a probe for EMSA (Figure 5.3). The 206/244mut oligo has the same substitution in the 210 to 238 region as the *nor-1::GUS* reporter construct 332*norL*mut. The substitution resulted in changes in 21 of the 28 nucleotides while maintaining the same GC ratio. With 206/244 as probe, two shifted complexes were identified with an SU1 protein extract. A 250 fold excess of unlabeled 206/244 as a competitor decreased the intensity of NorLbp while a 250 fold excess of unlabeled 206/244mut was a less effective competitor for NorLbp. Complex A appears to be due to non-specific binding because both 206/244 and 206/244mut were effective competitors. No specific protein complexes could be identified with the AFS10 protein extract and the 206/244 probe. In addition, no specific shifted complexes were identified with 206/244mut as a probe with either SU1 or AFS10 protein extracts (data not shown). NorLbp binds specifically to the *nor-L cis*-acting site and requires AflR either directly or indirectly for binding function.

A potential *cis*-acting site, CRE1, that may be involved in the upregulation of aflatoxin synthesis in response to cAMP, has been identified in the *nor-1* promoter (Dr. Ludimila Roze, personal communication). A similar site was identified upstream from

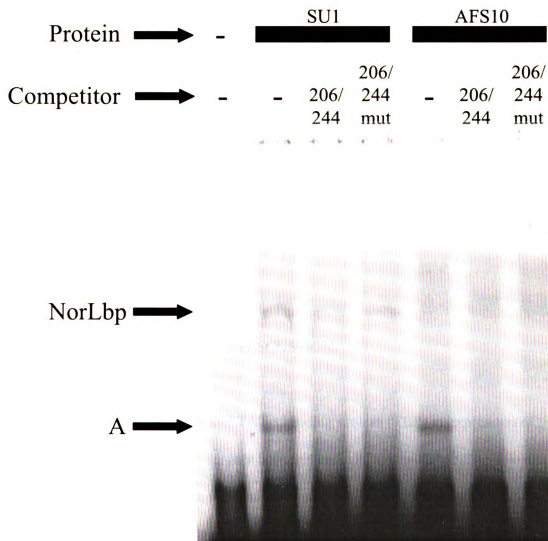


Figure 5.3. EMSA with a 206/244 oligo. 20 fmol of the 206/244 oligonucleotide was used as a probe with 32 μ g of protein extract from SUI or AFS10. NorLbp complex and complex A are indicated with arrows.

CRE1 and was named CRE2. Both CRE1 and CRE2 contain the core sequence: TGACAT a/g A. With CRE1 as probe, one shifted complex of similar migration was identified with both SU1 and AFS10 protein extracts (Figure 5.4). We have designated the protein responsible for the complex CREbp. To test for specificity of CREbp binding, competitions were performed with 250 fold excess of unlabeled CRE1, CRE1mut and CRE2 oligos. For both SU1 and AFS10 protein extracts, CRE1 was an effective competitor and CRE1mut was not. With the SU1 extract, CRE2 appears to only marginally compete for CREbp binding. No specific complexes were identified with CRE1mut, CRE2, and CRE2mut probes with either SU1 or AFS10 protein extracts (data not shown). CREbp binds specifically to CRE1 and appears to require AfIR for maximum complex formation.

DISCUSSION

The role of TATA boxes and TATA binding protein (TBP) in expression of aflatoxin biosynthesis genes has largely been ignored. While candidate TATA boxes have been previously identified, we demonstrated that the *nor-1* promoter TATA box is necessary to detect GUS activity. Transformants carrying the 332TATAmut *nor-1*::GUS vector had no detectable activity despite containing functional AfIR, CREbp and NorLbp binding sites. The *nor-1* TATA box needs to be present for these other *cis*-acting sites to be functional in terms of GUS activity.

Currently, only five aflatoxin biosynthetic genes have had their transcriptional start point experimentally determined (Figure 5.5). In Figure 5.5, the sixty bases upstream from the major transcriptional start point in each of these genes are displayed.

	-60	-50	-40	-30	-20	-10	+1
A	CACCGCCATA	<u>TATAGTGGGA</u>	TACGATCATG	GGTCTTTGGT	GGTTTCAACA	TTTCTTGAGT	A
B	TATCTAATAT	CAATTTATTA	<u>TCTTAGACCT</u>	CCTCATGCAA	CGGTGCTTCC	TTCTGCCAGT	G
C	ATCTCGAAGT	GTAGTTTTCA	AATACTGATA	<u>TAGCTTCCTA</u>	TAGCTCCCTCG	GGGCGGACC	T
D	CCGAGGAAAG	ATTTGTTTGG	TGGCCAACCA	TCCATAGCTG	<u>CGTATATATG</u>	TACTACATGC	C
E	GGGCCGGCTA	CTCTCCCGGA	GCAAGCCTTC	ACCTTGTGTG	TTTTCTTTCC	CGCTTTCAAT	T

Figure 5.5. Location of putative TATA boxes in *A. parasiticus* aflatoxin biosynthesis gene promoters. The experimentally determined major transcriptional start point is shown at +1. (A) *nor-1* (Trail et al., 1994) (B) *avnA* (Cary et al., 2000) (C) *pksA* (Ehrlich et al., 2002) (D) *ver-1* (Skory et al., 1992) (E) *aflR* (Ehrlich et al., 1999a).

The location of putative TATA boxes in *avnA* (Cary et al., 2000; Figure 5.5B), *pksA* (Ehrlich et al., 2002; Figure 5.5C) and *ver-1* (Skory et al., 1992; Figure 5.5D) are -40, -29 and -13 respectively. While the functionality of the *avnA*, *pksA* and *ver-1* TATA boxes is unknown, we hypothesize that the structural genes in the aflatoxin biosynthesis pathway contain functional TATA boxes. To test this hypothesis, the transcriptional start point needs to be identified in more aflatoxin genes and the TATA boxes need to be tested for function in the context of a wild type promoter. Substitution of a suspected *cis*-acting site in the context of a wild type promoter is a more rigorous test of function than a deletion.

While the four structural genes described above have putative TATA boxes in their promoters, the first 60 bp of the *aflR* promoter does not contain a sequence resembling a TATA box (Ehrlich et al., 1999a; Figure 5.5E). *AflR* is an aflatoxin biosynthetic pathway regulator and perhaps is regulated differently than the aflatoxin structural genes. As additional aflatoxin promoters are mapped, it will be interesting to see if *AflR* is unique in its lack of a TATA box.

The TATA binding protein (TBP) binds to the TATA box in eukaryotes. The TBP gene has been cloned from *A. nidulans* (Kucharski and Bartnik, 1997). The *A. nidulans* TBP promoter has consensus *cis*-acting sites for CreA (carbon repression), AreA (nitrogen repression) and AbaA (conidiophore development) (Kucharski and Bartnik, 1997). The levels of *A. nidulans* TBP mRNA varied several fold under diverse growth conditions that are consistent with the presence of CreA and AreA sites in the TBP promoter (Kucharski and Bartnik, 1997).

Previously, a potential *cis*-acting site was localized between -332 and -76 bp from the *nor-1* transcriptional start point (Miller et al., 2003a). Analysis of different sized

nor-1 promoters in *nor-1::GUS* strains further localized the potential *cis*-acting site to between -210 and -238. The *in vivo* significance of the 210 to 238 region (*norL*) was verified by analyzing isolates carrying a *nor-1::GUS* construct with *norL* replaced in the context of the 332 bp *nor-1* promoter fragment. While a functional *norL* site is not sufficient for *nor-1* transcriptional activation, it is necessary for maximum *nor-1* transcriptional activation in the context of the 332 bp promoter. Using EMSA, we demonstrated that NorLbp binds specifically to *norL*. In addition, NorLbp is dependent on AflR for binding activity, either directly or indirectly. The identity of NorLbp and whether other aflatoxin genes contain NorLbp *cis*-acting sites is unknown. Future work is focused on further defining the *cis*-acting site and cloning *norLbp*.

Another possible *cis*-acting site was recently identified, CRE1 (Dr. Ludmilla Roze, personal communication). Preliminary experiments demonstrated that CREbp binding to CRE1 and *nor-1* transcriptional activation are increased in the presence of cAMP in *A. parasiticus* solid cultures (Dr. Ludmilla Roze, personal communication). In addition, cAMP addition at high levels decreased protein kinase A (PKA) activity which coincided with decreased phosphorylation of CREbp (Dr. Ludmilla Roze, personal communication). We demonstrated that CREbp binds specifically to CRE1 in *A. parasiticus* liquid cultures and that AflR is necessary for maximum CREbp binding activity. The gene for CREbp has not been identified but efforts are underway to purify CREbp and in order to generate protein sequence data (Dr. Ludmilla Roze, personal communication). Interestingly, the *ver-1*, *pksA*, and *avnA* promoters do not have an identical match for CRE1 (TGACATAA). Nor-1 catalyzes an early step in the pathway and may be regulated differently than other aflatoxin structural genes. In addition,

further analysis of CRE1 may define the core CRE1 more specifically which may help identify CREbp *cis*-acting sites in other aflatoxin promoters.

By combining all the information regarding AfIR, NorLbp and CREbp, a model for *nor-1* transcriptional activation emerges (Figure 5.6). AfIR *cis*-acting sites are necessary for all aflatoxin genes analyzed including *nor-1* (Miller et al., 2003a), *pksA* (Ehrlich et al. 2002) and *avnA* (Cary et al., 2000). In addition, an AfIR site in the *aflR* promoter is necessary for *aflR* transcription (Ehrlich et al., 1999a). NorLbp appears to require functional AfIR for activity based on *in vitro* experiments but the mechanism is not known. Our experiments suggest that NorLbp is a transcriptional activator for *nor-1*. Confirmation of NorLbp function requires additional testing. CREbp appears to be only marginally affected by functional AfIR in *in vitro* experiments. CREbp bound to CRE1 under aflatoxin inducing conditions yet there is evidence that CREbp also binds under non-inducing conditions (Dr. Ludmila Roze, personal communication). Consequently, we can not determine if CREbp is an activator and/or repressor of *nor-1* transcription. Confirmation of CREbp function also requires additional testing. Protein kinase A (PKA) is activated by cAMP. Preliminary evidence suggests that phosphorylation of AfIR by PKA results in reduced AfIR function while phosphorylation of CREbp by PKA resulted in increased CREbp binding to CRE (Dr. Ludmila Roze, personal communication). Additional experiments are needed to clarify how phosphorylation of CREbp and AfIR by PKA affect their function.

This study is significant because it provides the first direct evidence for the existence of transcription factors other than AfIR in aflatoxin gene expression. Future

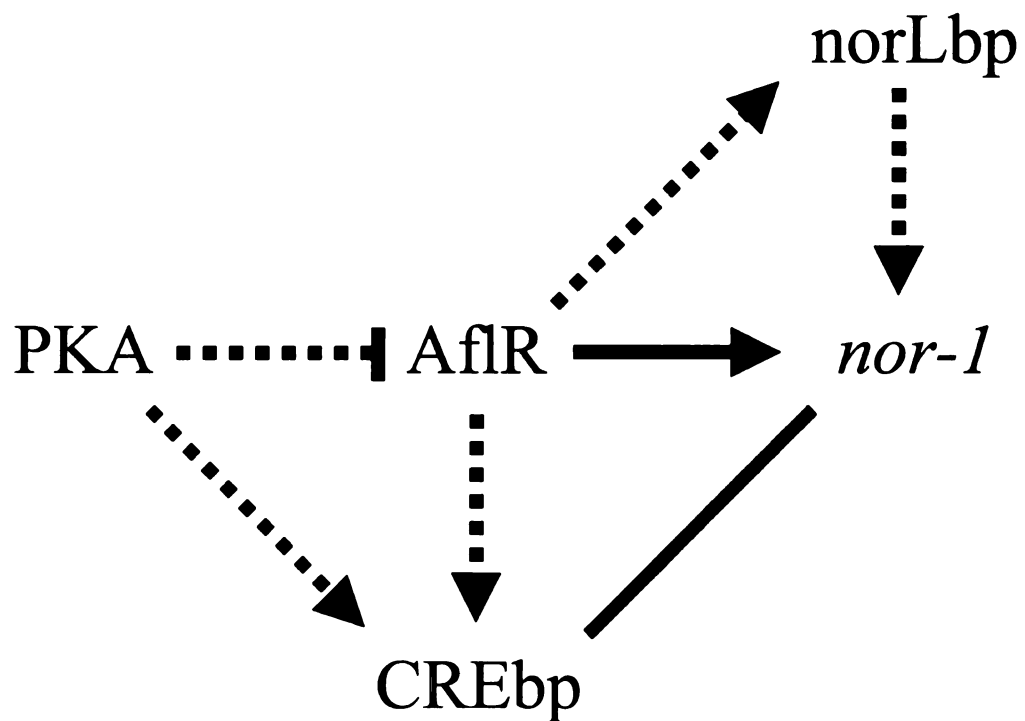


Figure 5.6. Proposed model for *nor-1* transcriptional activation. Arrows indicate positive interactions, blocked lines indicate negative interactions and lines with no block or arrow indicate unknown interaction. Definite interactions are represented by solid lines while inconclusive interactions are represented by dashed lines.

identification of the CREbp and norLbp genes will help verify their role in *nor-1* transcriptional activation. In addition, how CREbp and norLbp are regulated and what signals they respond to will aid our understanding of *nor-1* transcriptional regulation.

ACKNOWLEDGMENTS

All experiments described in Chapter 5 were performed by Michael Miller. The future publication status of this chapter is undecided.

CHAPTER 6

Future Studies

The studies described in this dissertation have provided a more detailed understanding of *nor-1* transcriptional regulation. However, several questions about *nor-1* transcriptional regulation remains. Confirmation of the functionality of CRE1 and norL *cis*-acting sites will require the identification of their respective binding proteins (CREbp and norLbp respectively). Once the binding proteins are cloned, experiments could be designed to explore how CREbp, norLbp and AfIR all interact to regulate *nor-1* transcription. In addition, we do not know if the CRE and norL *cis*-acting sites are unique to *nor-1*. Perhaps all the aflatoxin structural genes are regulated in a similar manner. However, the regulation of *nor-1* may have more levels of control than other aflatoxin structural genes because it catalyzes an early or critical step for aflatoxin biosynthesis.

The function of AfIR2, an AfIR binding site located approximately 1200 bp from *nor-1* transcriptional start site, in *nor-1* transcriptional activation is currently unclear because of the ORF3 gene located between AfIR2 and *nor-1*. A *nor-1* reporter plasmid that includes AfIR2 in the *nor-1* promoter may also include a transcriptionally competent and functional copy of ORF3. Integration of this reporter plasmid creates an extra copy of ORF3. ORF3 has no known function and sequence analysis has not provided any clear clues. However, an increased concentration of ORF3 protein may explain the higher *nor-1* transcriptional activation, especially if ORF3 is a transcriptional activator (CREbp or norLbp?). An ORF3 knock-out would provide preliminary evidence for

ORF3 function. In addition, a *nor-1* reporter plasmid that includes AflR2 but has a missense mutation in ORF3 would determine if AflR binding to AflR2 can directly activate *nor-1* transcription.

In filamentous fungi, the clustering of genes is a common feature of several metabolic pathways including mycotoxin biosynthesis. Others have postulated that the linkage of metabolic pathway genes provides a means to coordinately regulate pathway gene transcription, possibly via enhancers. Integration of a *nor-1* reporter plasmid outside of the aflatoxin gene cluster results in significantly lower *nor-1* transcriptional activation than integration within the aflatoxin gene cluster. Mechanisms for gene-cluster-dependent regulation in fungi are currently unknown. The aflatoxin gene cluster is an ideal model for future studies of cluster-dependent regulation for several reasons: 1) aflatoxin cluster is well characterized, 2) some strains of *A. parasiticus* (including NR1) have portions of the aflatoxin cluster duplicated elsewhere in their genome, and 3) several tools are already available including *ver-1* and *nor-1* reporter strains.

Determination as to whether the duplicated region of the aflatoxin gene cluster also has cluster-dependent regulation would help localize possible enhancers. The large spacer region at one end (the other end of aflatoxin cluster is currently undefined) of the aflatoxin gene cluster is an attractive target for future studies on cluster dependent regulation as well. Mechanistic analysis of aflatoxin gene-cluster-dependent regulation may help studies with other gene clusters as well.

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