Role of *cis*-Acting Sites *NorL*, a TATA Box, and *AflR1* in *nor-1* Transcriptional Activation in *Aspergillus parasiticus*

Michael J. Miller,[1,2]† Ludmila V. Roze,[1] Frances Trail,[3] and John E. Linz[1,2,4]*

Department of Food Science and Human Nutrition,[1] Center for Integrative Toxicology,[2] Department of Plant Pathology,[3] and National Center for Food Safety and Toxicology,[4] Michigan State University, East Lansing, Michigan

The transcription factor AflR is required for up-regulation of specific pathway genes involved in aflatoxin biosynthesis in the filamentous fungus *Aspergillus*. *nor-1* encodes an early aflatoxin pathway enzyme; its promoter contains a consensus AflR binding site (*AflR1*). Proteins in *Aspergillus parasiticus* cell extracts and AflR expressed in *Escherichia coli* do not bind to *A. parasiticus AflR1* in vitro, so it was not clear if this site was required for *nor-1* expression or if other transcription factors contributed to gene regulation. In this study we defined the role of AflR1 in *nor-1* expression in *A. parasiticus* and identified additional *cis*-acting sites required for maximum *nor-1* transcriptional activation. Deletion and substitution of *AflR1* in the *nor-1* promoter in *A. parasiticus nor-1*::GUS reporter strains showed that this site is required for *nor-1* transcriptional activation in vivo. Substitution of a putative TATA box in the *nor-1* promoter resulted in nondetectable β-glucuronidase (GUS) activity, demonstrating that this TATA box is functional in vivo. We also identified a novel *cis*-acting site, designated *NorL*, between residues −210 and −238 that was required for maximum *nor-1* transcriptional activation in *A. parasiticus* grown in liquid medium and on solid medium. Using an electrophoretic mobility shift assay, we identified a specific *NorL*-dependent DNA-protein complex that relies on a functional AflR, either directly or indirectly, for maximum binding capacity. Because the *NorL* site appears only once in the aflatoxin gene cluster, its association with the *nor-1* promoter may have important implications for the overall regulatory scheme for the aflatoxin pathway.

Aflatoxins, which are mycotoxins that are produced predominantly by *Aspergillus parasiticus* and *Aspergillus flavus*, frequently contaminate economically important crops, such as corn, cotton, peanuts, and tree nuts (31). Aflatoxin B$_1$, the most abundant of the aflatoxins, is also the most toxic and carcinogenic (21). Animal studies have demonstrated that aflatoxin is a potent hepatocarcinogen, and human epidemiological data have linked aflatoxin exposure with liver cancer (reviewed in references 11 and 17). As a result, susceptible crops are monitored for aflatoxin contamination in the United States and throughout the world, resulting in a large economic cost to growers and marketers of commodities. Our long-term goal is to reduce or eliminate aflatoxin from the food chain.

By elucidating the molecular mechanisms that regulate aflatoxin biosynthesis, we hope to generate novel approaches and targets for inhibition of aflatoxin gene expression. Aflatoxin biosynthesis is a complex process that requires at least 18 different enzymatic activities (3). The genes involved in aflatoxin biosynthesis reside in a 70-kb cluster and appear to be coregulated (30). Analysis of the regulatory mutant *A. flavus* strain 650 (2) first identified AflR as a pathway regulator (23). Subsequently, *aflR* homologs were identified in *A. parasiticus* (6) and *Aspergillus nidulans* (33).

Several independent lines of evidence confirmed that AflR has a key regulatory role in aflatoxin biosynthesis (6, 12, 13, 15, 16, 23, 32, 33). AflR *cis*-acting sites are required for transcriptional activation of three aflatoxin biosynthetic genes in vivo, including *stcU* (15), *avnA* (4), and *pksA* (14). Although AflR is a key regulator of aflatoxin synthesis, it is not clear that AflR is the only transcription factor required for transcriptional activation of all aflatoxin genes or if all of the consensus AflR *cis*-acting sites in the aflatoxin cluster are functionally significant.

We focused on the expression of *nor-1*, a gene encoding an enzyme that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin (29, 34). The *nor-1* promoter (Fig. 1) includes a consensus AflR binding site (*AflR1*; TCGNNNNNCGR) (14) located at positions −75 to −64 with respect to the primary *nor-1* transcriptional start site (position 1). Additional upstream consensus AflR binding sites are located at positions −1213 (*AflR2*) and −1563 (*AflR3*) (14). Genes upstream from *nor-1* include an open reading frame with an unknown function (*ORF3*; translational start at position −1073) and the divergently transcribed *pksA* gene (translational start at position −1731) (7, 30).

In previous studies, *A. parasiticus* nuclear extracts (13, 22) and AflR expressed in *Escherichia coli* (13) could not bind to *nor-1* promoter fragments containing *AflR1* in vitro, suggesting that *AflR1* might not be functionally relevant. Directed mutations in *AflR1* did not affect transcription of the divergently transcribed *pksA* gene in vivo (14), suggesting that *AflR1* is not important for expression of *pksA*. By using the same mutagenesis approach, both *AflR2* and *AflR3* were shown to be necessary for *pksA* transcription, but the effects of *AflR1*, *AflR2*, and

* Corresponding author. Mailing address: 234B GM Trout Food Science & Human Nutrition Bldg., Michigan State University, East Lansing, MI 48824. Phone: (517) 355-8474, ext. 126. Fax: (517) 353-8963. E-mail: jlinz@msu.edu.
† Present address: Department of Food Science, North Carolina State University, Raleigh, N.C.
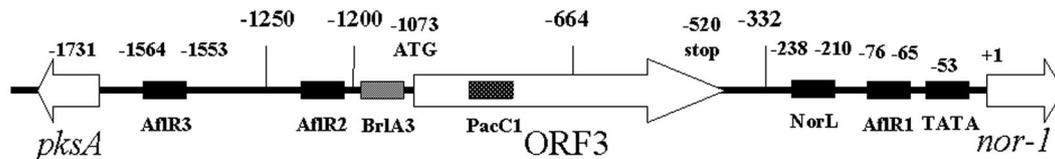
FIG. 1. Schematic diagram of the *A. parasiticus nor-1* promoter region. The numbers indicate the numbers of nucleotide residues upstream from the transcriptional start site. The positions of potential *cis*-acting sites are indicated, including the positions of *AflR1*, *AflR2*, and *AflR3* (AflR binding sites), *NorL*, and TATA, as well as *PacC1* and *BrlA3*, which were reported previously to be involved in *pksA* transcriptional regulation (14). The location of an open reading frame (*ORF3*) with an unknown function is also shown. The sizes of the *nor-1* promoters used in this study also are indicated (332, 76, and 64 bp).

AflR3 mutations on *nor-1* and *ORF3* expression were not reported (14). Furthermore, alteration of specific BrlA and PacC *cis*-acting sites in the *pksA–nor-1* intergenic region by directed mutagenesis significantly altered *pksA* transcription, indicating that AflR may not be the only transcriptional regulator for *pksA* (14).

Based on the previous data, we hypothesized that interaction of AflR with the *AflR1* site plays an important role in regulation of *nor-1* expression but that one or more additional transcription factors are required for formation of this protein-DNA complex.

The objectives of this study were (i) to clarify the role of AflR and *AflR1* in expression of *nor-1* in *A. parasiticus* and (ii) to identify additional *cis*-acting sites required for maximum *nor-1* transcriptional activation. This study demonstrated conclusively that multiple transcription factors (including AflR and NorLbp) are required for maximum *nor-1* promoter activity under our experimental conditions. Because the *NorL* site appears only once in the aflatoxin gene cluster, its association with the *nor-1* promoter may have important implications for the overall regulatory scheme for the aflatoxin pathway.

MATERIALS AND METHODS

Bacterial and fungal strains. *E. coli* DH5α F′ [φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r$_k$⁻ m$_k$⁺) *phoA supE44* λ⁻ *thi1 gyrA96 relA1*] (Invitrogen, Carlsbad, Calif.) was used to amplify plasmid DNA by standard procedures (1). *A. parasiticus* SU-1 (= ATCC 56775) was the wild-type aflatoxin-producing strain used in this study. *A. parasiticus* AFS10 is an *aflR* gene disruption strain (5) and does not produce aflatoxin. *A. parasiticus* NR-1 (*niaD*) (18) was the recipient strain used for plasmid transformation.

Plasmid constructs. Construction of plasmid pNANG-3 and pAPGUSNN-B has been described previously (10). To test the functionality of *cis*-acting sites, *nor-1* promoter fragments were generated by PCR by using pAPGUSNN-B as the template and primers with NotI (3′) and PacI (5′) tails. Promoter fragments were ligated into the NotI and PacI sites in pNANG-3, resulting in the β-glucuronidase (GUS) reporter plasmid series designated pBNGn (where n indicates the size of the promoter in base pairs).

(i) pBNG332 and pBNG332TATAmut. The polyadenylation site for *ORF3*, located 332 bp upstream from the transcriptional start site of *nor-1*, was used to define the 5′ end of the *nor-1* promoter. This 332-bp promoter fragment was amplified by PCR with primers JL267 (5′-GTTAATTAAGTCGAGCGGACATGGC CACG-3′; PacI site underlined) and JL186 (5′-TCGCGGCCGCTAAGTGATCC ATTCATTATGTC-3′; NotI site underlined). To generate pBNG332TATAmut, the TATA box was replaced by a PmeI site (5′-ATATATAG-3′ changed to 5′-GT TTAAAC-3′) in the context of the 332-bp *nor-1* promoter. The 332-bp *nor-1* promoter was divided into two PCR fragments (PacI/PmeI and NotI/PmeI) that were joined at the TATA box. The primers used for the PacI/PmeI fragment were JL267 and JL414 (5′-ATGTTTAAACTGGGATACGATCATGGGTC-3′; PmeI site underlined). The primers used for the NotI/PmeI fragment were JL186 and JL415 (5′-GGGTTTAAACGGCGGTGTGTTGGTCG-3′; PmeI site underlined). After

digestion with the appropriate restriction endonucleases, a three-fragment ligation was performed with pNANG-3, the PacI/PmeI fragment, and the NotI/PmeI fragment. The *nor-1* promoter in pBNG332 and pBNG332TATAmut was verified by nucleotide sequence analysis.

(ii) pBNG298, pBNG268, pBNG238, and pBNG210. To generate a *nor-1* promoter deletion series, different upstream PCR primers with PacI sites (underlined sequences) were used with the same downstream PCR primer (JL186 with a NotI site). The upstream primers used were JL411 (5′-CCTTAATTAAAACT GCTATGGTGACCTATTG-3′) for pBNG298, JL412 (5′-CATTAATTAACCA CATAGGCTACTCAAAAT-3′) for pBNG268, JL413 (5′-GGTTAATTAAAG ATCTCTGCTATTAAGTCGG-3′) for pBNG238, and JL302 (5′-CCCTTAAT TAATAGCGTGCTGGATGCGCGAA-3′) for pBNG210. The *nor-1* promoters in this deletion series were verified by nucleotide sequence analysis.

(iii) pBNGNorLmut. To generate pBNGNorLmut, a 28-bp region (positions −210 to −238) was replaced (changed from 5′-AGATCTCTGCTATTAAGTC GGTGATTAG-3′ to 5′-GTATAAGAAGTTTGTGATGGGATTCGTC-3′) in the context of the 332-bp *nor-1* promoter. The 332-bp *nor-1* promoter was divided into two PCR fragments (PacI/210 and NotI/238) that were joined at position −224. The primers used for the PacI/210 fragment were JL267 and JL613 (5′-CAAACTTCTTATACGCTCATGTCAATTTTGAG-3′). The primers used for the NotI/238 fragment were JL186 and JL612 (5′-TGATGGGAT TCGTCCGTGCTGGATGCGC-3′). JL612 and JL613 do not have restriction endonuclease sites. After digestion with the appropriate restriction endonucleases, a three-fragment ligation was performed with pNANG-3, the PacI/210 fragment, and the NotI/238 fragment. The *nor-1* promoter in pBNG332NorLmut was verified by nucleotide sequence analysis.

(iv) pAFLRMBP. The pMAL protein fusion and purification system (New England Biolabs, Beverly, Mass.) was used for AflR production by using methods described previously for Ver-1 (20). A DNA fragment encompassing the entire *AflR* open reading frame was generated by PCR by using an *aflR* cDNA fragment and PCR primers 5′-TTTCTAGAGTTGACCATATCTCCCCCC-3′ and 5′-CCAAGCTTTCATTCTCGATGCAGGTAAT-3′. The resulting product was cloned in frame into the XbaI and HindIII sites of expression vector pMALc2. Expression and purification of the AflR::MBP fusion protein (approximately 94 kDa) from *E. coli* were performed according to the manufacturer's instructions (New England Biolabs). Approximately 0.4 μg of fusion protein was included in electrophoretic mobility shift assay (EMSA) reaction mixtures as described below. A 40-fold excess of purified maltose binding protein (MBP) (16 μg) generated by using the same methods that were used for AflR::MBP was included in control reaction mixtures.

Generation and analysis of *A. parasiticus* transformants. Transformation of *A. parasiticus* protoplasts was performed as described by Horng et al. (18). Two to four micrograms of DNA and approximately 10⁷ protoplasts resulted in approximately 100 transformants. The site of plasmid integration was determined by PCR analysis (10) and was confirmed by Southern analysis. With the PCR assay, a 2.0-kb amplicon was diagnostic for 3′ *nor-1* integration. Genomic DNA was purified from *A. parasiticus* cultures shaken for 48 h in 100 ml of YES liquid medium (2% yeast extract, 6% sucrose; pH 5.8) at 29°C with five 6-mm-diameter glass beads (28) and was subjected to Southern hybridization analysis by using standard procedures (1). A 2.5-μg portion of genomic DNA was digested with ScaI and probed with a 900-bp ClaI fragment isolated from the *nor-1* terminator region of pAPGUSNN-B (10). Digestion of DNA from the recipient strain, NR-1, with ScaI generated a 3.0-kb restriction fragment, while a 3′ integrant (*nor-1* terminator) resulted in 3.7- and 4.0-kb restriction fragments.

GUS reporter assays. As an indirect measure of *nor-1* promoter function, GUS reporter activity (19) was measured in a liquid culture and a solid culture essentially as reported previously (10). The fluorescence of protein from the liquid culture was analyzed by using a Cytofluor II fluorimeter (Biosearch Co., Bedford, Mass.). The excitation and emission wavelengths were 360 and 460 nm, respectively. A standard curve for 4-methylumbelliferone (10 to 600 nM) in GUS lysis buffer also was generated, and GUS activity was expressed in picomoles of 4-methylumbelliferone produced per minute per milligram of protein.

EMSA. (i) Protein extraction. Cell protein extracts were generated by using modifications of the procedures of Peters and Caddick (25) and Peres-Esteban et al. (24). Conidiospores (1×10^7) were inoculated into 1-liter flasks containing 500 ml of appropriate growth medium and 10 glass beads. The cultures were incubated at 29°C for 48 h with shaking (150 rpm). Mycelia were collected by filtration by using Miracloth (Calbiochem, San Diego, Calif.), washed with sterile cold water, frozen with liquid nitrogen, and ground with a mortar and pestle. The ground mycelia were transferred to a 125-ml flask containing 5 ml of lysis buffer (25 mM HEPES-KOH [pH 7.5], 50 mM KCl, 5 mM MgCl$_2$, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 ml of fungal protease inhibitor cocktail [Sigma, St. Louis, Mo.] per 20 g of mycelia) and stirred on ice for 15 min. Saturated ammonium sulfate was slowly added to a final concentration of 10%, and the mixture was stirred for 15 min on ice and allowed to stand on ice for 15 min. Cellular debris was pelleted by centrifugation at $36,500 \times g$ for 30 min at 4°C. The concentration of ammonium sulfate in the supernatant was raised from 10 to 70% by adding solid ammonium sulfate slowly over 90 min with gentle stirring on ice. Cellular proteins were pelleted by centrifugation (10,000 × g for 20 min at 4°C), resuspended in dialysis buffer (15% glycerol, 15 mM HEPES-KOH [pH 7.9], 100 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 ml of fungal protease inhibitor cocktail [Sigma] per 20 g of mycelia), and dialyzed twice for 2 h each time (10,000-molecular-weight-cutoff dialysis membrane) in 2 liters of dialysis buffer. The protein concentration was determined by using the Bio-Rad reagent (Bio-Rad, Hercules, Calif.). The protein solution was divided into aliquots and stored at −80°C.

(ii) Probe generation. DNA probes were end labeled with [γ^{32}P]ATP by using Ready-to-Go kinase and following the manufacturer's instructions (Amersham, Piscataway, N.J.). The oligonucleotide probes and competitors used in EMSA for the NorL site were 206/244 (positions −244 to −205: ATG**AGCAGAT**C**TCTG** **CT**ATTAAGTCGGTGATTAG CGTGCT) and 206/244mut (positions −244 to −205: ATG**AGC**GTATAAGAAGTTTGTGATGGGATTCGTC CGTGCT) (the underlined residues represent the region from position −210 to position −238 designated *NorL*, and the residues in boldface type are part of a 14-base sequence with dyad symmetry). The probes used for analysis of the AflR site in the *nor-1* and *ver-1* promoter regions were *nor-1* AflR1 (5′-ACCGCCCAAC**TCG**GCCAGC**GAC** CAACACAC-3′; *AflR1* in boldface type) and *ver-1* AflR (5′-TCAGATAT**TCG**GT CTC**C**GAGGAAAGAT-3′; AflR in boldface type).

(iii) EMSA. EMSA and competition EMSA were performed as previously described (1). Binding reactions were performed at 30°C for 15 min by using the following components: 2 µg of nonspecific competitor DNA (polydeoxyinosinic-deoxycytidylic acid), 7.5 µg of bovine serum albumin, 20 fmol of labeled probe, and 32 µg of protein extract or 400 ng of the AflR::MBP fusion. When competitors were used, a 250-fold molar excess (5,000 fmol) of competitor was added to the binding reaction mixture. All components of the binding reaction mixture (including the competitor) were added to the tubes prior to addition of the protein extract.

RESULTS

In vitro AflR binding to *AflR1* in the *A. parasiticus* *nor-1* promoter. Recombinant AflR::MBP (full-length AflR fused to MBP and expressed in *E. coli*) did not form a stable complex with an oligonucleotide containing the *nor-1* *AflR1* site (Fig. 2) but did form a stable complex with an oligonucleotide containing the consensus AflR binding site in the *ver-1* promoter (Fig. 2). This complex was not detected when no protein or only MBP was added in the EMSA. These data are consistent with data from previous in vitro studies (13) that suggested that *A. parasiticus* AflR has little or no affinity for *AflR1* in *nor-1* but has high affinity for the AflR binding site in several *A. parasiticus* aflatoxin gene promoters, including the *ver-1* promoter.
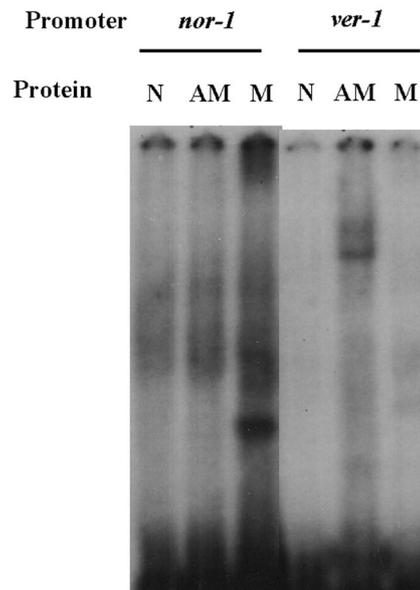

FIG. 2. EMSA of *AflR1* sites in *nor-1* and *ver-1* promoters. Twenty femtomoles of radiolabeled oligonucleotides carrying *AflR1* from the *nor-1* and *ver-1* promoters was subjected to EMSA in the presence of an AflR::MBP (AM) fusion protein (0.4 µg per reaction mixture), MBP (M) (as a control; 16 µg per reaction mixture), or no protein (N) (see Materials and Methods). The arrow indicates the location of the specific AflR::MBP/*ver-1* oligonucleotide complex.

***AflR1* and *nor-1* promoter activity in *A. parasiticus*.** Transformants carrying *nor-1*::GUS reporter plasmids with 332-, 76-, and 64-bp *nor-1* promoter fragments (*AflR1* located between positions −76 and −64) were constructed. Transformants carrying *nor-1*:GUS with a substitution mutation in *AflR1* (TCG GCCAGCGA changed to AGTTTAAACAG) in the context of the 332-bp promoter also were produced.

A. parasiticus NR-1 transformants were screened with a PCR assay to identify clones with a single copy of the reporter plasmid at the 3′ end of the *nor-1* gene. Single-copy integration at 3′ *nor-1* was subsequently confirmed by Southern blot analysis (10). This location is known to be important for correct regulation of *nor-1* promoter activity (10). The percentage of transformants with the correct integration ranged from 2 to 10% depending on the experiment (data not shown).

GUS activity, as an indirect indicator of *nor-1* promoter activity, was measured in duplicate for two confirmed 3′ *nor-1* integrants for each reporter construct in liquid shake cultures (glucose minimal salts medium, 48 h) and on solid growth medium (YES medium, 46 h) (26). In the liquid medium, two independent isolates carrying the wild-type 332-bp promoter fragment (isolates 332-1 and 332-2; wild-type *AflR1*) had easily measurable GUS activity (5.4 pmol/min/mg), while two independent isolates carrying the 332-bp AflRmut promoter fragment with an *AflR1* substitution in the context of the 332 promoter (AflR1m-1 and AflR1m-2) and the recipient strain NR-1 (control) had no detectable GUS activity. In agreement with these data, on solid medium, 332-1 and 332-2 showed clearly detectable GUS activity (blue color in colonies), while AflR1m-1 and AflR1m-2 and NR-1 (control) displayed no detectable GUS activity (Fig. 3A). Of particular interest, shorter promoter frag-

ments either with *AflR1* (76 bp) or without *AflR1* (64 bp) showed no detectable GUS activity (22). These data confirmed that *AflR1* is necessary but not sufficient for *nor-1* transcriptional activation in *A. parasiticus* and suggested that there is an important *cis*-acting site between positions −76 and −332.

Activity of the TATA box in the *nor-1* promoter of *A. parasiticus*. Two independent fungal isolates carrying the wild-type 332-bp *nor-1* promoter fragment and two independent isolates carrying the 332-bp *nor-1* promoter with a TATA substitution mutation (332TATAmut) in *nor-1*::GUS constructs were tested for GUS activity in liquid medium and on solid YES growth medium. Isolates with the TATA substitution mutation (isolates TATAm-1 and TATAm-2) and the recipient strain NR-1 (control) had no detectable GUS activity either in liquid medium or on solid growth medium (Fig. 3B), while isolates with the wild-type 332-bp *nor-1* promoter (isolates 332-1 and 332-2) had easily detectable activity in liquid GMS medium (5.4 pmol/min/mg) and on solid YES medium (Fig. 3B). These data confirmed that the TATA box is required (under the conditions tested) for *nor-1* transcriptional activation in vivo.

Role of *NorL* in *nor-1* transcriptional activity in *A. parasiticus*. Deletion analysis of the *nor-1* promoter described above suggested the presence of an important *cis*-acting site localized between positions −332 and −76. To localize this potential site, the GUS activities of two independent fungal isolates carrying *nor-1*::GUS constructs with 332-, 298-, 268-, 238-, and 210-bp promoter fragments were measured in liquid medium and on solid growth medium. Isolates carrying *nor-1*::GUS constructs with the 332-, 298-, 268-, and 238-bp promoter fragments all had easily detectable GUS activity in liquid medium (ranging from 1 to 13 pmol/min/mg) and on solid medium (Fig. 3C). The isolates with the 210-bp promoter (with a wild-type *AflR1* site) had no detectable activity in either medium (Fig. 3C), supporting the hypothesis that there is an important *cis*-acting site located between positions −210 and −238.

To verify the significance of the region from position −210 to position −238 (designated *NorL*) in vivo, two independent isolates carrying a *nor-1*::GUS construct with a *NorL* substitution mutation in the context of the 332-bp *nor-1* promoter fragment (NorLmut) were generated. In liquid medium, a *NorL* substitution mutation (isolates NorLm-1 and NorLm-2) resulted in a 2.5-fold reduction in GUS activity relative to the wild-type 332-bp promoter fragment (5.4 ± 2.2 pmol/min/mg) and a more-than-threefold reduction relative to the 238-bp promoter fragment (7.2 ± 5.8 pmol/min/mg). The variation between duplicate samples for the two independent isolates was relatively large in liquid medium. However, the data did demonstrate a clear trend, which was confirmed by GUS activity data derived from the same isolates grown on solid medium (Fig. 3C). Deletion or substitution of NorL in isolates NorLm-1 and NorLm-2 consistently and reproducibly reduced the GUS activity relative to the activities observed with the 332-, 298-, 268-, and 238-bp promoter fragments on YES solid medium. When a fluorescence assay was performed with extracts prepared from the same isolates grown on solid YES medium for 72 h, replacement of *NorL* reduced GUS activity approximately sixfold in NorLm-1 (isolate #124A) and approximately twofold in NorLm-2 (isolate #85) compared to the 332-bp wild-type *nor-1* promoter fragment (7 pmol/min/mg) (isolate #10A).
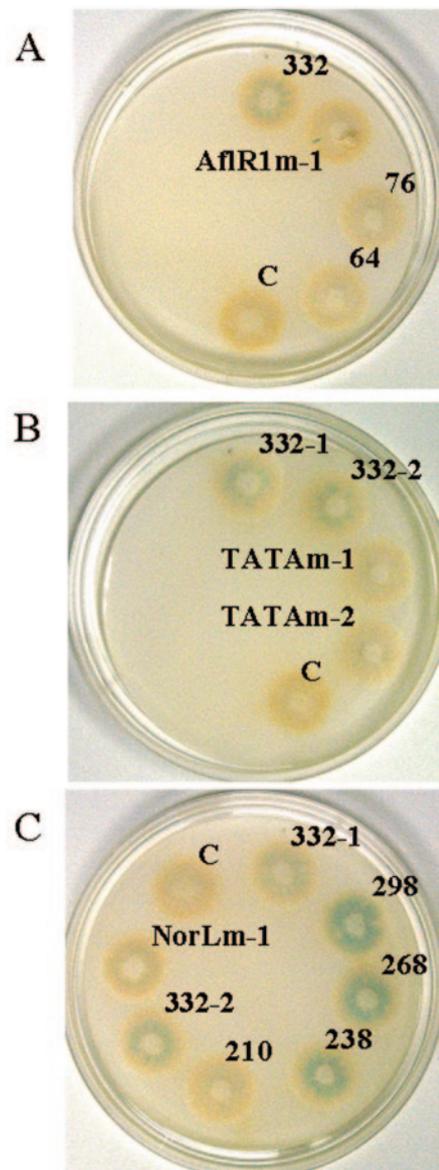

FIG. 3. Deletion and substitution analysis of the *nor-1* promoter. A GUS activity analysis was performed with two independent single-copy 3′ integrants from each *nor-1*::GUS reporter construct grown on YES solid medium as described in Materials and Methods. Representative colonies are shown as follows: in panel A, isolate 1 carrying the wild-type 332-bp promoter fragment (332), deletion mutants 76 and 64, isolate 1 carrying the 332-bp fragment with an *AflR1* substitution mutation (AflRm-1), and the control strain NR-1 (C) (no plasmid integrated into the genome); in panel B, isolates 1 and 2 carrying the wild-type 332-bp promoter fragment (332-1 and 332-2), isolates 1 and 2 carrying the 332-bp fragment with a TATA substitution mutation (TATAm-1 and TATAm-2), and the same control strain (C) as in panel A; and in panel C, isolates 1 and 2 carrying the wild-type 332-bp promoter fragment (332-1 and 332-2), deletion mutants 298, 268, 238, and 210, isolate 1 carrying the 332-bp fragment with a *NorL* substitution mutation (NorLm-1), and the same control strain (C) as in panel A.

In previous studies, exogenous cyclic AMP (cAMP) (5 mM) had a strong positive regulatory effect on *nor-1* promoter activity in *A. parasiticus* grown in liquid medium and on solid medium. This effect was mediated at least in part by a novel *cis*-acting site designated *CRE-1* (26, 27). We found that the
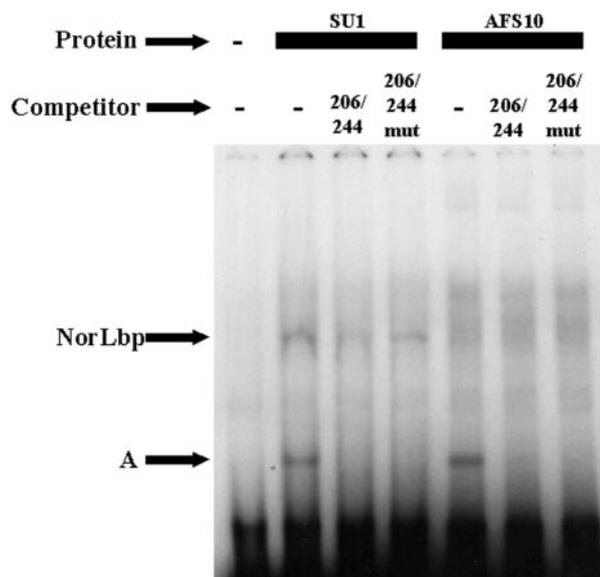
FIG. 4. EMSA of *NorL*/NorLbp DNA-protein complex in the *A. parasiticus nor-1* promoter. Twenty femtomoles of radiolabeled oligonucleotide 206/244 was used as a probe with 32 µg of protein extract from SU-1 or AFS10 grown under aflatoxin-inducing conditions. EMSA competitors (206/244 and 206/244mut) were used in a 250-fold excess. The positions of DNA-protein complexes containing NorLbp and protein A are indicated by arrows.

relative ability of the *nor-1* promoter to respond to exogenous cAMP was not affected in either NorLm-1 or NorLm-2, which was consistent with the observation that the cAMP response is mediated by *CRE-1* (27). Thus, while a functional *NorL* site is not sufficient for *nor-1* transcriptional activation, it is necessary for maximum *nor-1* transcriptional activation under a variety of growth conditions.

DNA-protein complexes with *NorL*. To detect DNA binding proteins that interact with *NorL*, we performed EMSA with a radiolabeled, 40-bp, wild-type oligonucleotide probe (206/244) or a mutant derivative (206/244mut) covering the region from position −210 to position −238 (*NorL*) and cell extracts from strains SU-1 (wild type) and AFS10 (AflR knockout) grown under aflatoxin-inducing conditions. Extracts from each strain were prepared on two independent occasions and gave similar EMSA results. The 206/244mut oligonucleotide had the same substitution in the region from position −210 to position −238 as the *nor-1*::GUS reporter construct 332NorLmut. The substitution changed 21 of 28 nucleotides while maintaining the G+C content.

With the wild-type 206/244 oligonucleotide probe, two shifted complexes carrying DNA binding proteins designated NorLbp and protein A were identified with an SU-1 protein extract (Fig. 4). A 250-fold excess of unlabeled wild-type probe 206/244 effectively competed with the NorLbp/DNA complex and the protein A/DNA complex, suggesting that both complexes represent specific DNA-protein interactions. However, a 250-fold excess of unlabeled probe 206/244mut was not an effective competitor for the NorLbp/DNA complex but prevented protein A/DNA complex formation. These data confirmed that NorLbp participated in a specific complex whose formation was mediated by the 28-bp region designated *NorL*.

NorL substitution mutations resulted in two- to sixfold reductions in the *nor-1* promoter activity complex, supporting this conclusion. The NorLbp/DNA complex was not observed with the wild-type 206/244 oligonucleotide probe and AFS10 protein extracts (Fig. 4) or with the 206/244mut oligonucleotide probe and either SU-1 or AFS10 protein extracts (data not shown). We concluded that a specific DNA/protein (NorLbp) complex depends on *NorL* for formation and requires AflR either directly or indirectly for maximum binding capacity. We did not identify the number of proteins in the NorLbp/DNA complex.

DISCUSSION

Experiments with *AflR* null mutants and AflR-inducible expression strains along with AflR-DNA binding studies demonstrated that AflR is required for aflatoxin biosynthesis and the expression of specific aflatoxin pathway genes. For example, transcriptional activation of the middle pathway gene *avnA* occurs through a single consensus *cis*-acting site (4). An additional potential AflR binding site (TCGNNNNNCGR) in the *avnA* promoter, when mutated, did not alter *avnA* transcription in vivo, and AflR protein expressed in *E. coli* was unable to bind to this nonfunctional binding site in vitro (4). The *stcU* promoter has three consensus AflR binding sites within 800 bp of the translational start site (15). Mutational analysis of the three AflR binding sites revealed that the most distal site, at position −762, had no effect on *stcU* transcription, while AflR consensus binding sites at positions −81 and −168 both appeared to be functional in vivo (15). The activities of the two functional AflR binding sites were not additive, as *stcU* promoters with either the position −81 site or the position −168 site were indistinguishable from strains that had both AflR binding sites (15).

In contrast, EMSA with *A. parasiticus* AflR expressed in *E. coli* 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* than for the consensus AflR binding sites in several other aflatoxin biosynthetic promoters (13; this study), casting doubt on the functional significance of *AflR1*. We tested this hypothesis directly by analyzing the effects of substitution mutations on *nor-1* promoter function using *nor-1*::GUS reporter constructs. Substitution of *AflR1* in the 332-bp *A. parasiticus nor-1* promoter resulted in nondetectable levels of GUS activity, confirming that *AflR1* is necessary for *nor-1* transcriptional activation in *A. parasiticus* under the growth conditions utilized in this study.

The case for AflR being the sole regulator of all aflatoxin biosynthesis structural genes is not as strong. For example, we found that a TATA box and a *CRE-1* site (27) play important roles in regulation of *nor-1* promoter activity. AflJ also has been putatively assigned a role as a transcriptional coactivator (8) and has been reported to interact directly with AflR (9). Furthermore, studies with the *pksA* promoter (14) provided evidence that both the PacC (pH sensing) and BrlA (sporulation) regulatory proteins can alter *pksA* transcriptional regulation through *cis*-acting sites in the *pksA*–*nor-1* intergenic region (Fig. 1). However, in preliminary experiments, deletion of the PacC and BrlA consensus *cis*-acting sites did not affect *nor-1* transcriptional regulation in *A. parasiticus* under the con-

ditions tested (22). These data suggest either that *nor-1* does not require the PacC and BrlA sites for transcriptional activation or that these sites are not required under our specific growth conditions.

In the present study, analysis of *A. parasiticus* isolates carrying deletions in the *nor-1* promoter resulted in the important conclusion that although AflR is necessary for *nor-1* expression, it is clearly not sufficient for maximum expression. The data also demonstrated that at least one additional *cis*-acting site(s) located between positions −332 and −76 contributes to *nor-1* transcriptional activation. There are no additional consensus AflR binding sites (TCGNNNNNCGR) in this promoter region. Therefore, one or more unknown transcriptional activators presumably bind in this promoter region and influence *nor-1* transcriptional activation in *A. parasiticus*. Based on deletion analysis, there is a *cis*-acting site, *NorL*, in the region from position −210 to position −238 of the *nor-1* promoter. Substitution of *NorL* reduced *nor-1* promoter activity two- to sixfold in liquid growth medium and on solid growth medium, confirming the biological significance of this site. Mutation of *NorL* did not influence the relative ability of the *nor-1* promoter to respond to cAMP, which is consistent with the role of *CRE-1* in mediating the cAMP regulatory effect (27). EMSA identified two specific complexes containing DNA binding proteins, designated NorLbp and protein A, that formed when a 40-bp oligonucleotide probe carrying the *NorL* site was used; both complexes were effectively competed with wild-type probe, but only the protein A/DNA complex was competed with a 40-bp mutant oligonucleotide probe sequence. The presence of the protein A/DNA complex and the absence of the NorLbp/DNA complex as determined by EMSA when the AFS10 cell extract (*aflR* knockout) was used suggested that formation of the protein A/DNA complex is independent of *aflR* expression, while formation of the NorLbp/DNA complex depends on *aflR*.

The wild-type 40-base oligonucleotide probe used in EMSA contained a 14-bp sequence that is a candidate for the *NorL* *cis*-acting site: 5′-**AGCAGA**TC**TCTGCT**-3′ (the nucleotides in boldface type display dyad symmetry). Based on the location of this site in the *NorL* region, we propose that NorLbp may be a heterodimer that forms around the 14-bp *NorL* site. Protein A (AflR independent and part of a faster-migrating complex) could be one protein in the pair that binds to either *NorL* half-site, 5′-AGCAGA-3′. Consistent with this hypothesis, the NorLmut oligonucleotide, which retains four of six bases in this 5′ half-site (**AGC**GTA; retained bases are indicated by boldface type), can compete with wild-type oligonucleotide for protein A/DNA complex formation. The putative *NorL* site is present only in the *nor-1* promoter and not in the promoters of other genes in the aflatoxin pathway. Thus, the regulation of *nor-1* could be unique, which would not be totally unexpected given the function of the Nor-1 protein in the first committed step of the aflatoxin biosynthetic pathway. In preliminary experiments, deletion of a 50-bp fragment containing *AflR2* (located immediately upstream of *ORF3*) greatly reduced *nor-1* promoter activity (22). These results could be explained if *ORF3* encodes the AflR-dependent component of NorLbp.

Deletion analysis of the *nor-1* promoter also suggested the presence of a negative regulator in the region from position −298 to position −332. Deletion of this region consistently

increased GUS activity driven by the *nor-1* promoter both in liquid growth medium and on solid growth medium. Although this region was not the focus of the present study, we will conduct a more thorough analysis of this region to identify this additional putative transcription factor binding site.

The variation in *nor-1* promoter activity in *A. parasiticus* was greater in liquid shake cultures than in cultures grown on plates. GUS activity on solid medium was more consistent and reproducible for independent isolates on the same plate and for plates in the same experiment. Although differences in promoter activity could be due to a change in medium composition, we think that differences in the growth environment are more important. In submerged liquid shake cultures, asexual sporulation is not induced, and the organism grows in pellets that are very different from the fungal colonies observed on plant material. Growth on a solid surface more closely mimics the natural growth environment, where the fungus grows as a mycelial mat and sporulates asexually. A more detailed analysis of aflatoxin promoter function during growth in liquid medium and on solid medium is needed to resolve this issue.

A potential problem with many aflatoxin promoter studies is that the integration site for the reporter constructs is not in the aflatoxin gene cluster (e.g., *trpC* for *stcU*::GUS [15] and *niaD* for *avnA*::GUS [4]). If *ver-1*::GUS was integrated at *niaD*, then the promoter activity was >500-fold less than if it was integrated at *ver-1* (20), even though the timing of transcription was the same. A similar effect has been reported for the *pyrG* locus (10). In the present study, the *A. parasiticus nor-1*::GUS reporter constructs were integrated at the *nor-1* locus, which is critical to the normal timing and level of reporter transcription in a pattern similar to the wild-type *nor-1* gene pattern (10).

Our objective in this study was to determine if the AflR1 site in the *nor-1* promoter was functional or if other transcription factors played a role in *nor-1* regulation. Using EMSA (in vitro) and functional mutation analysis (in vivo) of the *nor-1* promoter, we clearly demonstrated that *AflR1* is required but not sufficient for *nor-1* transcriptional activation in *A. parasiticus*. We also showed that a TATA box and a unique and novel *cis*-acting site (*NorL*; located between residues −210 and −238) in the *A. parasiticus nor-1* promoter are necessary for maximum transcriptional activation in vivo. Finally, a specific *NorL*-dependent DNA-protein (NorLbp) complex was identified that relies on a functional AflR, either directly or indirectly, for maximum binding activity. These data strongly suggest that regulation of *nor-1* is important in the overall regulatory scheme for aflatoxin synthesis, in keeping with the important role of Nor-1 in catalyzing the synthesis of the first stable pathway intermediate.

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