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Increased Expression of *Aspergillus parasiticus aflR*, Encoding a Sequence-Specific DNA-Binding Protein, Relieves Nitrate Inhibition of Aflatoxin Biosynthesis

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The *aflR* gene from *Aspergillus parasiticus* and *Aspergillus flavus* may be involved in the regulation of aflatoxin biosynthesis. The *aflR* gene product, AFLR, possesses a GAL4-type binuclear zinc finger DNA-binding domain. A transformant, SU1-N3(pHSP), containing an additional copy of *aflR*, showed increased transcription of *aflR* and the aflatoxin pathway structural genes, *nor-1*, *ver-1*, and *omt-1*, when cells were grown in nitrate medium, which normally suppresses aflatoxin production. Electrophoretic mobility shift assays showed that the recombinant protein containing the DNA-binding domain, AFLR1, bound specifically to the palindromic sequence, TTAGGCCTAA, 120 bp upstream of the AFLR translation start site. Expression of *aflR* thus appears to be autoregulated. Increased expression of aflatoxin biosynthetic genes in the transformant might result from an elevated basal level of AFLR, allowing it to overcome nitrate inhibition and to bind to the *aflR* promotor region, thereby initiating aflatoxin biosynthesis. Results further suggest that *aflR* is involved in the regulation of multiple parts of the aflatoxin biosynthetic pathway.

Aflatoxins are a family of toxic, polyketide-derived metabolites from *Aspergillus flavus* and *Aspergillus parasiticus*. The recent advances in our and other laboratories in cloning several aflatoxin pathway genes (6, 7, 23, 28, 31, 32, 35) revealed that most of these genes are clustered within a 60-kb DNA region in *A. parasiticus* and *A. flavus* (33, 36). This finding has spawned renewed interest in the study of the regulation of aflatoxin biosynthesis. Previous studies have suggested that one of these genes, *aflR*, is involved in some aspect of the regulation of aflatoxin biosynthesis (6, 28). The *aflR* gene product, AFLR, contains a GAL4-type binuclear zinc finger cluster, Cys-$(Xaa)_2$-Cys-$(Xaa)_6$-Cys-$(Xaa)_6$-Cys-$(Xaa)_2$-Cys-$(Xaa)_6$-Cys (6, 34). The $(Cys)_6$-type structure is a distinct feature of some of the pathway-specific regulatory proteins in fungi and yeasts (4, 16, 37). In addition, AFLR contains a highly acidic domain adjacent to the carboxyl terminus (this study), similar to that found in GAL4 and GCN4 (15, 21). In *Saccharomyces cerevisiae*, this region is necessary for the DNA-binding protein's ability to activate transcription of other pathway related genes (16, 21). Payne et al. (28) showed that transformation with *aflR* of a mutant of *A. flavus* blocked in production of all aflatoxin pathway precursors with *aflR* restored it to aflatoxin proficiency. Wild-type and blocked *A. parasiticus* strains, on transformation with *aflR*-containing vectors, overproduced aflatoxin precursors in aflatoxin permissive medium, such as potato dextrose broth and Adye and Mateles medium (6). Aflatoxins or precursors were also produced by these transformants when cells were grown in nitrate medium, which normally inhibits aflatoxin production (6). Interestingly, sclerotial morphogenesis was also affected in these *aflR* transformants grown in nitrate medium (unpublished observations).

Nitrogen regulates the biosynthesis of secondary metabolites in a variety of microorganisms. Nitrate represses the production of polyketide-derived mycotoxins in *A. parasiticus* and *Alternaria alternata* (1, 17, 25, 26). Niehaus et al. (25) suggested that repression by nitrate is due to an increased cytoplasmic NADPH/NADP ratio, which favors biosynthetic reduction, promoting utilization of malonyl coenzyme A and NADPH for fatty acid synthesis, rather than for polyketide synthesis. Other researchers suggested that nitrate represses formation of enzymes involved in polyketide synthesis (17, 26). In this report, we show that nitrate inhibits transcription of aflatoxin pathway genes in *A. parasiticus*, including *aflR*, *nor-1* (32), *ver-1* (31), and *omt-1* (35) but that the transcription of these genes is restored in transformants containing an additional copy of *aflR*.

MATERIALS AND METHODS

Fungal strains. *A. parasiticus* SU-1 (ATCC 56775), a wild-type aflatoxigenic strain, and SU1-N3(pHSP), a derivative of SU-1 that was transformed with the *aflR*-containing vector pHSP (see Fig. 3A), were maintained on potato dextrose agar (Difco, Detroit, Mich.) or grown on 5/2 agar (5% V8 juice, 2% agar [pH 5.2]) for the production of conidia.

Isolation of total RNA. *A. parasiticus* mycelia grown in parallel for 48, 72, 96, and 120 h in Cove's minimal salt medium (9) supplemented with 10 mM nitrate (nitrate medium) were collected on Miracloth, blotted dry, quickly frozen with liquid nitrogen, and stored at −80°C until use. Mycelia were ground to a fine powder in a mortar and pestle in the presence of liquid nitrogen. Total RNA was isolated by the hot phenol extraction protocol (24).

Northern (RNA) hybridization analysis. Twenty micrograms of total RNA per sample was fractionated in a 0.4 M formaldehyde–1.2% agarose gel and transferred to a GeneScreen Plus membrane (Dupont NEN Research Products, Boston, Mass.) for probing with [α-^{32}P]dCTP-radiolabeled DNA probes prepared by using the Random Primed DNA Labeling Kit (U.S. Biochemical Corp., Cleveland, Ohio).

Cloning and sequencing of *aflR* cDNA. A cDNA library in lambda phage vector was constructed with a directional ZAP-cDNA synthesis kit (Stratagene,

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La Jolla, Calif.). Screening of the cDNA library was performed with [α-^{32}P]dCTP-labeled probes generated from a 1.9-kb *Bam*HI-*Bam*HI genomic DNA fragment (Fig. 1). In vivo excision of pBluescript SK− phagemids from positive Uni-ZAP XR lambda clones was performed with the ExAssist/SOLR system (Stratagene) by a helper phage coinfection protocol. Several positive cDNA clones were isolated and analyzed with restriction enzyme digestion. The clone containing the largest cDNA insert was sequenced by the dideoxy chain termination method with Sequenase version 2.0 (U.S. Biochemical Corp.). The *aflR* cDNA sequence including the 5′ upstream sequence and the deduced amino acid sequence are shown in Fig. 1.

Determination of *aflR* copy number in transformants. The copy number of the *aflR* transformants was determined by a quantitative slot hybridization technique with a GeneScreen Plus nylon membrane. The SU1-N3 and SU1-N3(pHSP) genomic DNAs were denatured in 1 ml of 0.25 M NaOH–0.5 M NaCl for 10 min at room temperature. Four 200-μl portions of denatured DNA were loaded into individual sample wells of the apparatus (PR 648 slot blot; Hoefer Scientific Instruments, San Francisco, Calif.). After the DNA solution was drawn through the wells by vacuum, the wells were rinsed three times with 0.5 ml of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.125 N NaOH. The membrane was neutralized in 0.5 M NaCl–0.5 M Tris-HCl (pH 7.5), air dried, and cut in half. The blots containing duplicate DNA samples were probed, respectively, with ^{32}P-labeled *nor-1* and *aflR* DNA probes. The bound radioactivity was assayed by Cerenkov counting with an LS1800 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). The *nor-1* probe was included to normalize the counts for variability in amounts of DNA used. After normalization, the ratio of transformant *aflR* to SU-1 *aflR* was determined.

PCR. PCR was performed by using Ampli*Taq* DNA polymerase and a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Genomic DNA was heated at 94°C for 2 min and cycled 30 times through standard amplification procedures: denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 2.5 min, with a final extension step at 72°C for 5 min.

Expression and purification of recombinant AFLR. The *Sma*I-*Xho*I digest of *aflR* cDNA (residues 23 to 659) was ligated to *Sma*I-*Xho*I-digested pET29c (Novagen, Madison, Wis.) to give the plasmid pAFLR1. This construct has in-frame codons for six histidines at the C-terminal side of the *aflR* insert. The plasmid was transformed into *Escherichia coli* BL21(DE3)pLysS, and expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM to log-phase *E. coli* cells grown in Luria broth (LB) medium containing kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml). Expression of the fusion protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in aliquots taken at hourly intervals. The cells were recovered by centrifugation at 3,000 × *g* for 5 min, washed once with 50 mM Tris-HCl (pH 8)–2 mM EDTA, pelleted again, and then frozen at −80°C until use.

After thawing, the cells from a 30-ml culture were resuspended in 10 ml of 20 mM Tris-HCl–150 mM NaCl–0.1% Triton X-100–0.1 mM phenylmethylsulfonyl fluoride (pH 7.5), and lysis was allowed to proceed at 30°C for 15 min. The sample was sonicated briefly to decrease viscosity before centrifugation at 12,000 × *g* for 20 min. The fusion protein in 1 ml of this cellular extract was separated from most *E. coli* proteins by binding to 50 μl of nickel-nitriloacetic acid-Sepharose 4B (NiNTA-agarose; Qiagen, Chatsworth, Calif.) for 30 min at ambient temperature. After washing the NiNTA-agarose three times with 20 mM Tris-HCl–500 mM NaCl–5 mM imidazole (pH 7.9), the recombinant AFLR was recovered from the NiNTA-agarose by elution with 100 μl of 20 mM Tris-HCl–500 mM NaCl–200 mM imidazole (pH 7.9).

PAGE. Protein electrophoresis involved gels containing 12.5% polyacrylamide (29:1, acrylamide–bis-acrylamide), 0.1% SDS, and 0.375 M Tris-HCl (pH 8.8) and buffer containing 0.05 M Tris–0.38 M glycine–0.1% SDS. Native gels for electrophoretic mobility shift assays (EMSAs) were 10% polyacrylamide (5% when the 387-bp *aflR* fragment was used) in 0.25× TBE (1× TBE is 90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA).

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer by using standard cyanophosphoramidite chemistry. Oligonucleotides were purified on 20% polyacrylamide–7 M urea gels as previously described (18). For mobility shift assays, 10 pmol of one strand was radiolabeled with [γ-^{32}P]ATP in a reaction catalyzed by T4 polynucleotide kinase and then annealed to a fivefold excess of the unlabeled complementary strand. The oligonucleotides used in these studies are listed in Table 1. Preparation of the 387-bp *aflR* fragment containing the region upstream of the *aflR* translation start site involved digestion of the plasmid pBB (6) with *Bam*HI and *Eco*RI and purification of the fragment on a low-melting-point 1.2% agarose gel. Labeling of this fragment was done by filling in the *Bam*HI site in the presence of [α-^{32}P]dCTP and the Klenow fragment.

EMSA. Binding to the 387-bp *Bam*HI-*Eco*RI fragment of *aflR* and to the radiolabeled duplex oligonucleotides was done essentially as previously described (18) by using 1 to 5 μl of protein solution (0.12 to 0.60 μg of protein as measured by Coomassie G250 dye binding with bovine serum albumin as the standard) in 30 μl of buffer containing 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 10% glycerol, 10 μg of bovine serum albumin, 0.1 μg of poly(dI · dC), 5 mM dithiothreitol, and 10 μM zinc acetate. Competitor DNAs or oligonucleotides were allowed to incubate with protein at room temperature for 10 min prior to the addition of the radiolabeled duplex oligonucleotide. The binding mixture was

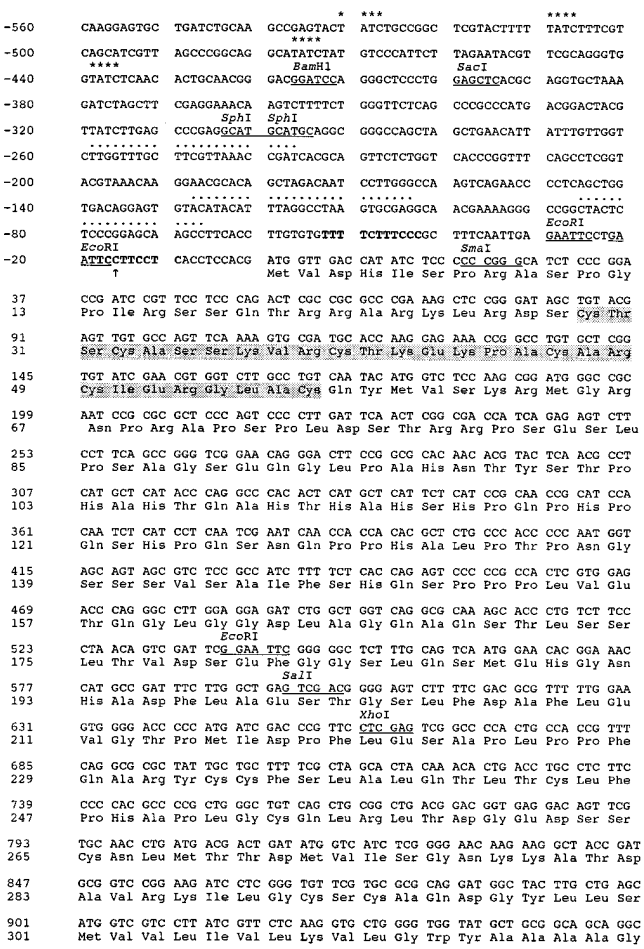

FIG. 1. Nucleotide sequence of *aflR* and its upstream region. Pyrimidine-rich stretches upstream of the translation start site are shown in boldface type. Several palindromic or partially palindromic stretches of DNA in the region upstream of the translation start site are indicated by dots, and putative GATA (TATC) binding sites are indicated by asterisks. The vertical arrow indicates the start of the *aflR* cDNA sequence. The deduced amino acid sequence is shown below the DNA sequence. The binuclear zinc finger domain is shaded. The acidic amino acid residues at the carboxyl terminus are doubly underlined. Two potential polyadenylation signal sequences are indicated by diamonds.

TABLE 1. Oligonucleotides used for EMSA

Oligonucleotide duplex ligand	Sequences[b]	Origin	Source or reference
AF-1[a]	5′-CTACT**CTCC**C**GGAG**CAAGCC-3′ 3′-GATGA**GAGG**G**CCTC**GTTCGG-5′	−67 to −86 in *A. parasiticus aflR* promoter	This study (Fig. 1)
AF-2	5′-ACATACAT**TTAGGCCTAA**GTGCGAG-3′ 3′-TGTATGTA**AATCCGGATT**CACGCTC-5′	−104 to −128 in *aflR* promoter	This study (Fig. 1)
AF-3	5′-TT**GGTTT**GCTTCGTT**AAACC**GAT-3′ 3′-AA**CCAAA**CGAAGCAA**TTTGG**CTA-5′	−237 to −259 in *aflR* promoter	This study (Fig. 1)
AF-4	5′-ACATACAT**TTA**CGCC**TAA**GTGCGAG-3′ 3′-TGTATGTA**AAT**GCGG**ATT**CACGCTC-5′	Same as AF-2 with position −115 changed from G to C	
NF-1	5′-AATTCCTTATTTTGGATTGAAG*CCAAT*ATGATAATGAGG-3′ 3′-TTAAGGAATAAAACCTAACTTC*GGTTA*TACTATTACTCC-5′	Contains binding site for NF-1	30
Sp1	5′-CGACCATAGT*CCCGCCCC*AAC*TCCGCCCATCCCGCCCCTAA-3′* 3′-GCTGGTATCA*GGGCGGGG*TTGA*GGCGGGTAGGGCGGGGATT-5′*	Contains binding sites for Sp1	2
CREB	5′-CTAGCGGC***TGACGTCA***TCAAG-3′ 3′-GCCG***ACTGCAGT***AGTTCGATC-5′	Contains binding site for CREB	11
GAL4	5′-T**CCGGAGGACTGTCCTCCGG**T-3′ 3′-A**GGCCTCCTGACAGGAGGCC**A-5′	Yeast GAL4-binding site	29

[a] Position in relationship to the translation start site.
[b] Palindromic or partially palindromic regions are indicated in bold. The DNA recognition sites of known DNA-binding proteins are shown in italics.

loaded onto native polyacrylamide gels after the addition of 3 μl of 1.5% bromophenol blue in 50% glycerol and electrophoresed at 25 mA until the blue dye ran most of the way down the gel. DNA-protein complexes were visualized by autoradiography.

Nucleotide sequence accession number. The GenBank accession number for the *aflR* sequence identified in this study is L26222.

RESULTS

Aflatoxin gene transcripts in wild-type and *aflR*-transformed *A. parasiticus* grown in nitrate medium. To correlate expression of aflatoxin pathway genes and the restoration of aflatoxin production in *aflR* transformants grown in nitrate medium, we used Northern hybridization analysis to examine the transcript levels of *nor-1*, *ver-1*, *omt-1*, and *aflR* from wild-type and *aflR*-transformed *A. parasiticus* [SU-1 and SU1-N3(pHSP)] grown in parallel in nitrate medium. Figure 2 shows that the transcripts of these genes from SU-1 were almost undetectable during the entire 5-day period, indicating that nitrate suppression of aflatoxin biosynthesis was at the transcriptional level. In contrast, the *aflR* transformant, SU1-N3(pHSP), showed higher levels of transcripts for the aflatoxin pathway genes than did SU-1. The level of the *aflR* transcript in SU1-N3(pHSP) peaked at 72 h and decreased slightly thereafter. The transcripts of *nor-1*, *ver-1*, and *omt-1* were detected at 96 h, approximately 24 h after the onset of *aflR* expression. The lag probably represents the time needed for AFLR production and subsequent activation of transcription of the other structural genes. The levels of *nor-1*, *ver-1*, and *omt-1* transcripts increased concomitantly and substantially during the 96- to 120-h incubation period whereas that of *aflR* decreased markedly.

Intactness of the *aflR*-containing segment from pHSP after integration into transformants. To determine the intactness of the segment containing the *aflR* gene from pHSP in the transformants, we used PCR to amplify a specific region (Fig. 3A, bracket 2) with a pair of primers, one derived from the sequence 90 bp from the *Sph*I site (Fig. 1) and another from the bacterial cloning vector pUC18. Genomic DNAs of three phenotypically identical *aflR* transformants, Ta, Tb, and Tc (that is, production of bullet-shaped sclerotia and golden yellow mycelia on potato dextrose agar plates and in Adye and Mateles medium) and of the recipient strain SU1-N3 were used in the PCR. Figure 3B shows that the bands obtained by PCR from the three transformants' genomic DNA were the same as those expected on the basis of a similar PCR with pHSP. Also, no such PCR products were generated from SU1-N3 genomic DNA. In addition, the 3-kb region of the *niaD* vector adjacent to *aflR* in pHSP (Fig. 3A, bracket 1) in the transformants was amplified. These results suggest that the transforming *aflR* and its 5′ upstream region remained intact. Southern hybridization

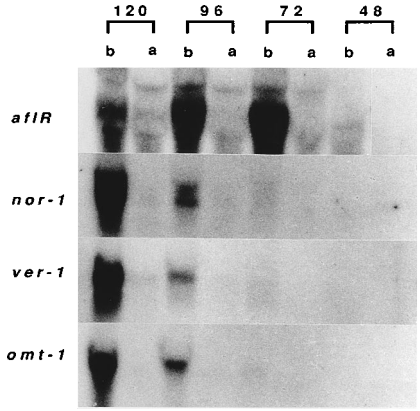

FIG. 2. Transcript levels of *aflR* and three aflatoxin pathway structural genes, *nor-1*, *ver-1*, and *omt-1*, in nitrate medium at different times. Northern blots with 20 μg of total RNA were probed with either a 1.9-kb *Bam*HI-*Bam*HI fragment of *aflR* (Fig. 1), a 0.7-kb *Xho*I-*Xho*I fragment of *nor-1* cDNA (32), a 1.0-kb *Eco*RI-*Xho*I fragment of *ver-1* cDNA (31), or a 1.2-kb *Eco*RI-*Eco*RI fragment of *omt-1* cDNA (35). The strains used were *A. parasiticus* SU-1 (lanes a) and SU1-N3(pHSP) (lanes b).

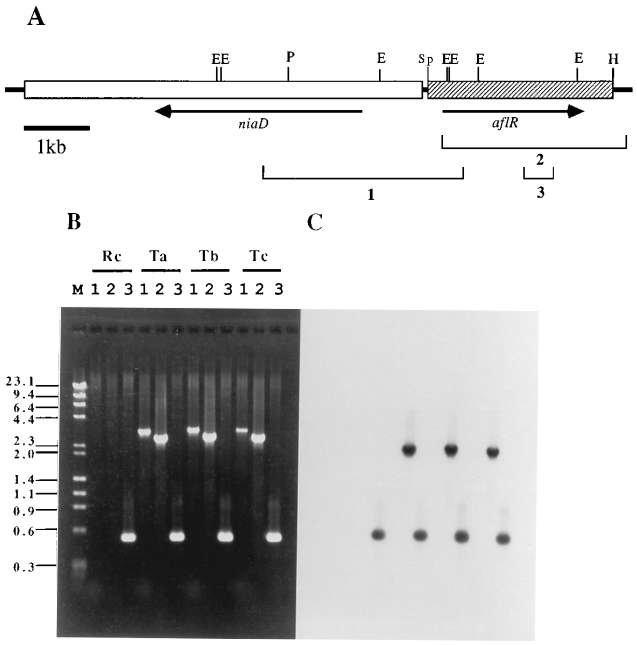

FIG. 3. (A) Restriction endonuclease map of pHSP used in the transformation. Abbreviations: H, *Hin*dIII: E, *Eco*RI; P, *Pst*I; Sp, *Sph*I. The schematic diagram represents the pUC18 sequence. The open box is the *niaD*-containing fragment. The hatched region is the *aflR*-containing fragment. The PCR-amplified regions corresponding to lanes 1 to 3 in panel B are indicated by brackets numbered 1 to 3, respectively. (B and C) Intactness of upstream region of *aflR* and *aflR* open reading frame in pHSP transformants. (B) PCR amplification of genomic DNA of the recipient strain, *A. parasiticus* SU1-N3 (Rc), and three transformants (Ta, Tb, and Tc). The primers used in the PCR were 5′-TCTAC CCTGGTAACCCTACG-3′ (derived from *niaD*) and 5′-TCTGATGGTCGC CGAGTTGA-3′ (reverse primer: positions 245 to 226 [Fig. 1]) (lanes 1), 5′-TCGGTACGTAAACAAGGAAC-3′ (forward primer: −206 to −195 [Fig. 1]) and 5′-CCAGTCACGACGTTGTAAAA-3′ (derived from pUC18) (lanes 2), and 5′-CCGATTTCTTGGCTGAGT-3′ (forward primer: 581 to 598 [Fig. 1]) and 5′-TCCTCATCCACACAATCC-3′ (reverse primer: 1100 to 1083 [Fig. 1]) (lanes 3). (C) Southern hybridization with a probe generated from *aflR* cDNA position 1040 to 1520 region. The blot hybridized with a probe generated from the *niaD* 1.4-kb *Eco*RI-*Pst*I fragment (Fig. 3A) is not shown.

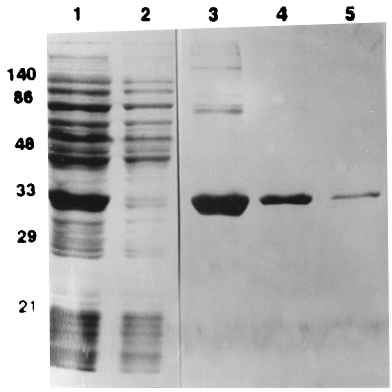

FIG. 4. SDS-PAGE gel showing purification of AFLR fusion protein by NiNTA-agarose. Lanes: 1, unpurified extract of IPTG-induced *E. coli* B21(DE3) pLysS transformed with pAFLR1 in 50 mM Tris-HCl–2 mM EDTA–1% Triton X-100 (pH 8.0); 2, the same extract as described for lane 1 after treatment with NiNTA-agarose; 3 to 5, aliquots of sequential 200-μl washes of the NiNTA-agarose with 20 mM Tris-HCl–500 mM NaCl–500 mM imidazole (pH 8.0). The locations of bands for molecular weight marker proteins (Bio-Rad) are indicated (in thousands) on the left.

at high stringency indicated that these PCR products were sequence specific for both *aflR* (Fig. 3C) and *niaD* (data not shown). The *aflR* copy number in the transformants was estimated from the ratio of transformant *aflR* to SU1-N3 *aflR*. The calculated ratios from the three transformants were in the range of 1.35 to 1.50. Recent studies suggest that *A. parasiticus* SU-1 contains duplicate copies of at least part of the aflatoxin gene cluster, since two copies of the *ver-1* and *aflR* genes have been identified (33). Therefore, these ratios and the results from PCR amplifications (Fig. 3A, bracket 2, and 3B, lanes 2, 5, 8, and 11) show that the transformants contain at least one additional copy of *aflR*. The expression of *aflR* at a higher level in transformants is not caused by the sequence upstream of *niaD* in pHSP (Fig. 3A), since transformation with the construct pHAC, in which the *niaD*-containing fragment in pHSP was repositioned at the *Hin*dIII site, gave the same kinds of transformants as those from pHSP (unpublished data). These results suggest that increased expression of *aflR* in the transformants was not due to the position at which integration occurred, but rather from the presence in the transformants of an additional copy of *aflR*.

Expression of AFLR DNA-binding domain in *E. coli*. The predicted AFLR protein contains a GAL4-type zinc finger cluster. Studies of other GAL4-type transcription regulatory proteins have shown that both the $[Zn(II)]_2(Cys)_6$ cluster and adjoining Arg- and Lys-rich regions are necessary for DNA-binding specificity (16, 29). Plasmid pAFLR1 was designed so that the fusion protein (AFLR1) contained the putative zinc-binding domain (amino acids 29 to 56), the adjacent Arg- and Lys-rich region (amino acids 57 to 79), and a $(His)_6$ domain at the C-terminal end of the protein to allow purification on NiNTA-agarose. The size of recombinant protein AFLR1 was approximately 32 kDa in SDS-PAGE (Fig. 4, lanes 3 to 5), which is in agreement with the size expected on the basis of the predicted amino acid composition. In the absence of IPTG, no such band was induced (results not shown), further indicating that the 32-kDa band results from expression of the recombinant gene.

AFLR is a sequence-specific DNA-binding protein. We first tested for binding of AFLR1 to part of the *aflR* promoter region. A ^{32}P-labeled 387-bp upstream segment of *aflR* (Fig. 1, −30 to −416) gave a discernible band shift in the presence of 0.1 μg of poly(dI · dC) (Fig. 5A). Because of the considerable homology of the AFLR zinc finger cluster to those of other GAL4-type proteins, we hypothesized that the DNA recognition site for AFLR would also be similar. Most of the GAL4-type recognition elements have dyad symmetry with a GC-rich region as an essential part of the binding domain (29). A computer search for palindromic or partially palindromic sites in the 5′ upstream region of *aflR* revealed several possible candidates (Fig. 1). To test whether AFLR1 could bind to these sites, we prepared three synthetic double-stranded oligonucleotides containing these sites (Table 1). Figure 5B showed the results of EMSA with oligonucleotides containing such sites from −67 to −259 bp (Fig. 1). Oligonucleotide AF-2 showed a band shift, indicating formation of DNA-protein complexes (Fig. 5B, lane 1). In contrast, oligonucleotides AF-1 and AF-3 did not bind to AFLR1 under these conditions (Fig. 5B, lanes 2 and 3). Also, these latter two oligonucleotides did not compete with ^{32}P-labeled AF-2. (Fig. 5C, lanes 1 and 2), although self-competition occurred (Fig. 5C, lane 3). AF-4, containing the same sequence as AF-2 but with the G at position 12 changed to a C, did not bind to AFLR1 (Fig. 5B, lane 4). No competition was found when a large excess (5 pmol) of unlabeled single-strand oligonucleotide (the upper strand of AF-2) was added to the binding reaction mixture (Fig. 5C, lane

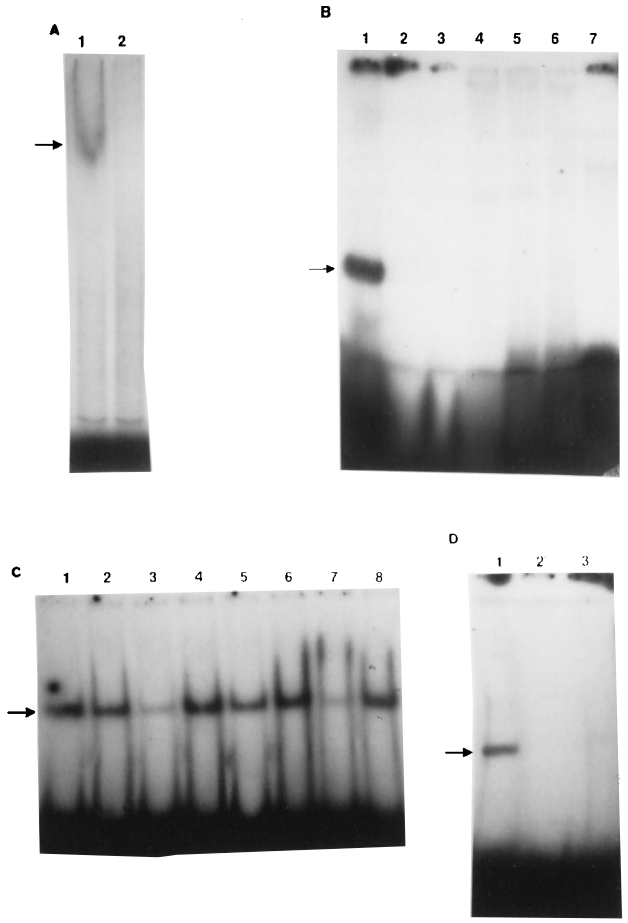

FIG. 5. (A) EMSA with ^{32}P-labeled 387-bp fragment of *aflR* upstream region and NiNTA-agarose-purified AFLR1. Binding reaction mixtures contained 0.6 μg of protein and 1 ng of DNA with 0.1 μg of poly(dI · dC) as the competitor (lane 1) or DNA only (lane 2). (B) EMSA with NiNTA-agarose-purified AFLR1 and 50 fmol of different ^{32}P-labeled oligonucleotides. Oligonucleotides were labeled to the same specific activity, and an equal amount (24,000 cpm) of each was used. Each reaction mixture contained 0.3 μg of AFLR1 and 1 μg of poly(dI · dC) as the nonspecific competitor. Lanes: 1, oligonucleotide AF-2; 2, AF-1; 3, AF-3; 4, AF-4; 5, CREB; 6, GAL4; 7, NF-1. (C) Effect of different competitors in EMSA with NiNTA-purified AFLR1 and 50 fmol of ^{32}P-labeled oligonucleotide AF-2. Only the 5′-to-3′ strand of oligonucleotide AF-2 was ^{32}P labeled. The competitors (1 pmol, unless otherwise noted) were AF-1 (lane 1), AF-3 (lane 2), AF-2 (lane 3), 5′-to-3′ strand of oligonucleotide AF-2 (5 pmol) (lane 4), CREB (lane 5), Sp1 (lane 6), and *M. lysodeikticus* DNA cut with *Taq*I (1 μg) (lane 7); no competitor was added in lane 8. In each lane, the lower band contained unbound oligonucleotide AF-2 while the upper band contained oligonucleotide-protein complexes. (D) EMSA with ^{32}P-labeled AF-2 and different *E. coli* extracts. Extracts were prepared as described in Materials and Methods, and the purification step with NiNTA-agarose was included. Each extract (5 μl) was used in the binding reaction mixtures. Lanes: 1, extract from IPTG-induced *E. coli* B21(DE3)pLysS transformed with pAFLR1; 2, extract from *E. coli* B21(DE3)pLysS; 3, extract from *E. coli* B21(DE3)pLysS transformed with pAFLR1 and grown to the same optical density as cells for lane 1 but without IPTG.

4). In spite of its homology to GAL4 in the zinc finger cluster, AFLR1 did not bind to oligonucleotides containing the GAL4 recognition core sequence (Fig. 5B, lane 6). AFLR1 also did not bind to sites for two other common DNA-binding proteins, CREB and Sp1, containing leucine zipper and $(Cys)_2$-$(Cys)_2$ DNA-binding motifs, respectively (Fig. 5B, lanes 5 to 7, and 5C, lanes 5 and 6). However, the GC-rich DNA from *Micrococcus lysodeikticus* (67 mol% GC content), competed to the same extent as unlabeled AF-2 for binding to ^{32}P-labeled AF-2 (Fig. 5C, lane 7), consistent with the GC-rich nature of the putative binding domain.

To demonstrate that only recombinant AFLR1 binds AF-2, we tested, by EMSA, extracts of *E. coli* B21(DE3)pLysS prepared in the same way as the recombinant AFLR1 (Fig. 5D). No complex formation was detected with the *E. coli* extract (Fig. 5D, lane 2) or with the extract of *E. coli* harboring pAFLR1 prior to IPTG induction (Fig. 5D, lane 3). These results demonstrate that the binding to AF-2 is indeed specific for AFLR1 and not a result of an *E. coli* DNA-binding protein present in the extract.

DISCUSSION

The AFLR recognition site that we found in the *aflR* upstream region is different from recognition sites previously identified for GAL4-type regulatory proteins found in *Aspergillus nidulans* (ALCR), *Neurospora crassa* (QA-1F), and *S. cerevisiae* (HAP1, LEU3, ARGRII, and GAL4) (see reference 10 and references therein). However, like most other GAL4-type recognition sites, the AFLR recognition sequence contains C's and G's in the palindromic portion that appear to be essential for binding, since replacement of a single G (oligonucleotide AF-4) in the palindrome prevented binding. The fact that AFLR binds to a site within its own promoter region suggests that *aflR* expression is autoregulated in a manner similar to that of other regulatory genes that encode metabolic pathway activators and that are part of a gene cluster, for example, *alcR* in *A. nidulans* (19), *qa-1F* in *N. crassa* (27), and *QUTA* in *A. nidulans* (3).

Increased expression of *nor-1*, *ver-1*, and *omt-1* in nitrate medium might result from an elevated basal level of AFLR due to increased *aflR* copy number in the transformants, allowing it to overcome the suppressive effect of nitrate on aflatoxin pathway gene transcription. Increased levels of AFLR would be available to bind to its own promoter sites and thereby activate the transcription of *aflR*. Activation of transcription could be modulated by the highly acidic domain (Fig. 1, TEERVLHHP SMVGEDCVDEEDQPRVADSLF) as has been established for GAL4 (TTGMFNTTTMDDVYNYLFDDEDTPPNPK KE) and GCN4 (15, 20, 21). Preliminary data have shown that AFLR1 also binds to sites in the promoter regions of several aflatoxin biosynthetic genes and may thereby activate their transcription (12).

Northern analysis indicates that nitrate suppresses aflatoxin biosynthesis at the transcriptional level. A similar mechanism might explain why the alternariol-*O*-methyltransferase activity, responsible for the formation of the polyketide mycotoxin alternariol monomethyl ether in *A. alternata*, disappears when cells are grown in medium containing nitrate as the sole nitrogen source (26). Nitrate assimilation in fungi involves the expression of a global regulatory gene, such as *areA* in *A. nidulans*, *nit-2* in *N. crassa*, and *nre* in *Penicillium chrysogenum* (5, 14), and a pathway-specific gene, such as *nirA* in *A. nidulans* and *nit-4* in *N. crassa* (4, 37). The global regulatory gene encodes a $(Cys)_2$-type zinc finger DNA-binding protein whereas the pathway-specific gene encodes a GAL4-type zinc finger activator which mediates induction of nitrate reductase and nitrite reductase. A recent DNA-binding study of *P. chrysogenum* penicillin biosynthetic genes *penAB* and *pcbC* by Marzluf's group showed that the global regulatory protein NIT2 binds to the intergenic region of *penAB* and *pcbC* (13). Since NIT2, AREA, and NRE have 98% identity in their DNA-binding domains, that is, the zinc finger and the adjacent basic region (14), these results suggest that structural genes for secondary metabolism are possibly regulated as members of a

nitrogen control circuit. A search of the *aflR* upstream region revealed several GATA . . . GATA motifs (−430 to −530), similar to those identified as binding sites for NIT2 and AREA (5, 8). These GATA core elements are approximately 300 to 400 bp away from the AFLR recognition site TTAGGCCTAA. If a NIT2- or AREA-like protein is present in *A. parasiticus* and binds to the GATA elements, the suppressive effect of nitrate on the transcription of aflatoxin pathway genes may be due to such an interaction with AFLR or with other protein factors regulating nitrogen metabolism.

The similarity of the carboxyl-terminal highly acidic regions (the last 30 amino acids; described above) of AFLR and yeast GAL4 suggests that, like GAL4, AFLR may bind to a specific GAL80-like negative regulator (22). If such negative regulation occurs for aflatoxin biosynthesis in *A. parasiticus* grown in medium containing nitrate as the sole nitrogen source, increased amounts of AFLR may be needed to inactivate the negative regulator from the nitrogen control circuit. We are currently investigating whether such control circuits occur in the regulation of polyketide biosynthesis.

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