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Molecular Characterization of *aflR*, a Regulatory Locus for Aflatoxin Biosynthesis

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Aflatoxins belong to a family of decaketides that are produced as secondary metabolites by *Aspergillus flavus* and *A. parasiticus*. The aflatoxin biosynthetic pathway involves several enzymatic steps that appear to be regulated by the *afl2* gene in *A. flavus* and the *apa2* gene in *A. parasiticus*. Several lines of evidence indicate that these two genes are homologous. The DNA sequences of the two genes are highly similar, they both are involved in the regulation of aflatoxin biosynthesis, and *apa2* can complement the *afl2* mutation in *A. flavus*. Because of these similarities, we propose that these two genes are homologs, and because of the ability of these genes to regulate aflatoxin biosynthesis, we suggest that they be designated *aflR*. We report here the further characterization of *aflR* from *A. flavus* and show that *aflR* codes for a 2,078-bp transcript with an open reading frame of 1,311 nucleotides that codes for 437 amino acids and a putative protein of 46,679 daltons. Analysis of the predicted amino acid sequence indicated that the polypeptide contains a zinc cluster motif between amino acid positions 29 and 56. This region contains the consensus sequence Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys. This motif has been found in several fungal transcriptional regulatory proteins. DNA hybridization of the *aflR* gene with genomic digests of seven polyketide-producing fungi revealed similar sequences in three other species related to *A. flavus*: *A. parasiticus*, *A. oryzae*, and *A. sojae*. Finally, we present evidence for an antisense transcript (*aflRas*) derived from the opposite strand of *aflR*, suggesting that the *aflR* locus involves some form of antisense regulation.

Aflatoxins are toxic and carcinogenic secondary metabolites produced by two filamentous fungi, *Aspergillus flavus* and *A. parasiticus*. These compounds have been studied extensively since the early 1960s, when they were shown to cause Turkey X disease (26). Studies since that time have shown aflatoxin B1, the predominate aflatoxin produced by these fungi, to be one of the most potent carcinogens known (36). Because of the potential health hazards to animals and humans consuming aflatoxins in food sources, research in several laboratories has been directed at elucidating the biosynthetic pathway. A biosynthetic scheme for aflatoxin production has been proposed (2, 11), and several laboratories are cloning genes for aflatoxin biosynthesis (4, 5, 12, 27, 35). Three pathway genes, *nor1*, *ver1*, and *omt1*, have been cloned from *A. parasiticus* (5, 35, 41).

In *A. flavus*, the *afl2* gene has been cloned and appears to regulate several steps in the pathway (27). This gene was isolated by complementation of a fungal strain blocked in aflatoxin biosynthesis at the *afl2* allele. Genetic studies with this strain have shown that a mutation in *afl2* prevents accumulation of norsolorinic acid, the first stable intermediate in the pathway (27). Further, metabolite feeding studies have shown that a functional *afl2* allele is required for the conversion of four known pathway intermediates to aflatoxin (27). Finally, studies with cell extracts have shown that a functional *afl2* allele is required for the enzymatic activity of three reactions known to occur in the pathway (27). Thus, the biochemical evidence indicates that *afl2* is a regulatory gene for aflatoxin biosynthesis.

Additional support for a regulatory role of *afl2* comes from the research of Chang et al. (4). They identified a gene in *A. parasiticus* that, when transformed into a wild-type strain, caused up-regulation of aflatoxin biosynthesis and accumulation of pathway intermediates. No mutation comparable to *afl2* is known in *A. parasiticus*, but sequence comparison of the *apa2* gene with the *afl2* gene has shown it to have greater than 96% sequence identity to *afl2*. Furthermore, we have shown that *apa2* complements the *afl2* mutation in *A. flavus* (39). Therefore, *apa2* appears to be a homolog of *afl2*. Because of the biochemical evidence supporting a regulatory role for *afl2* and the conserved DNA sequence for fungal regulatory genes, we propose that this gene and its homolog in *A. parasiticus*, *apa2*, be named *aflR* to designate its function as a regulatory gene for aflatoxin biosynthesis.

In this report, we present further characterization of the *aflR* locus in *A. flavus*. We show by sequence analysis of genomic and cDNA clones that *aflR* codes for a protein, AFLR, that has a zinc cluster DNA-binding motif homologous with several known fungal regulatory genes. We also show that a gene similar to *aflR* is present in the closely related species *A. oryzae* and *A. sojae*. Finally, we provide evidence that the *aflR* locus also encodes an antisense transcript.

MATERIALS AND METHODS

Fungal strains. *A. flavus* NRRL 3357 (wild type) was obtained as previously described (40). *A. oryzae* NRRL 3485, *A. sojae* NRRL 5594, *A. parasiticus* NRRL 2999, and *Penicillium griseofulvum* NRRL 994 and NRRL 1951 were obtained from the National Center for Agricultural Utilization Re-

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search in Peoria, Ill. *A. nidulans* 1079 and *A. parasiticus* SU-1 were obtained from the Southern Regional Research Center, New Orleans, La. *Cercospora kikuchii* PR was obtained from R. G. Upchurch, North Carolina State University, Raleigh. *Cochliobolus heterostrophus* was obtained from J. M. Daly, University of Nebraska, Lincoln.

Isolation and analysis of RNA and DNA. Total RNA was extracted from lyophilized mycelia of wild-type *A. flavus* by the procedure of Williamson et al. (37). Poly(A)$^+$ RNA was isolated by using oligo(dT)-cellulose chromatography (23). For Northern (RNA) analysis, 10 μg of poly(A)$^+$ RNA was electrophoresed through a 1.0% agarose gel containing 1.5% formaldehyde, transferred to a Zeta-Probe membrane (Bio-Rad, Richmond, Calif.), and hybridized with ^{32}P-labeled DNA probes. Fungal genomic DNA isolations and Southern analyses were done as previously described (38). Double-stranded sequencing was performed on both DNA strands by using the dideoxy-chain termination method (31). DNA analysis and database searches were performed by using the GeneWorks software program (Intelligenetics, Mountain View, Calif.). Additional database searches were done via Blast (National Center for Biotechnology Information).

Construction and screening of the cDNA library. In this study, two cDNA libraries (A and B) were constructed. Synthesis and cloning of both cDNA libraries into the Unizap vector were accomplished in accordance with the manufacturer's protocols (Stratagene, La Jolla, Calif.). The cDNA libraries were subsequently screened by plaque hybridization techniques (23) using a ^{32}P-labeled 2.8-kb *Bam*HI fragment from genomic clone GAP3-BB (see Fig. 1). In cDNA library A, cultures of *A. flavus* NRRL 3357 were grown initially in YES medium (2% yeast extract plus 6% sucrose) at 28°C on a rotary shaker (150 rpm) for 72 h. Mycelia were rinsed with 0.85% KCl, blended thoroughly, collected, and rinsed again. The mycelia were transferred to flasks containing peptone mineral salts (PMS) medium, a medium that does not support high levels of aflatoxin (3). The flasks were incubated for 24 h at 28°C on a rotary shaker (150 rpm). Mycelial mats were collected and rinsed with 0.85% KCl, divided into 2.5-g aliquots, and transferred to SLS medium (28) for induction of aflatoxin. Cultures were incubated at 28°C on a rotary shaker (100 rpm), and at various intervals one flask of each medium was sampled and the filtrates were analyzed for aflatoxin. Concurrently, mycelial mats were removed by vacuum filtration, immediately frozen with liquid nitrogen, lyophilized, and stored at −80°C. Under these conditions, aflatoxin first appears in the medium in 8 h and rapidly increases to a peak at 24 h (27). Poly(A)$^+$ RNA was isolated from frozen, lyophilized cultures sampled at 16, 20, and 24 h after transfer to SLS medium. The RNA was pooled and used to generate the cDNA library.

For cDNA library B, conidia (10^4) of *A. flavus* NRRL 3357 were inoculated into 9.5 ml of PMS medium in petri dishes (100 by 15 mm). After 3 days of growth at 28°C, the resulting mycelial mats were resuspended with 15 ml of PMS medium plus glucose (PMS-G) (3). At 18 h after addition of the PMS-G, mycelial mats were harvested and processed as described for library A. Poly(A)$^+$ RNA isolated from the mats was used to construct cDNA library B.

Aflatoxin analysis. The aflatoxin concentration in the medium was determined by high-pressure liquid chromatography analysis (40) and by enzyme-linked immunoassay with aflatoxin B1 monoclonal antibodies and an aflatoxin B1-horseradish peroxidase conjugate (7) kindly provided by J. Peska (Michigan State University).

Nucleotide sequence accession numbers. The GenBank accession number for *A. flavus aflR* and flanking sequences is L32577. The *aflR* cDNA accession number is L32576.

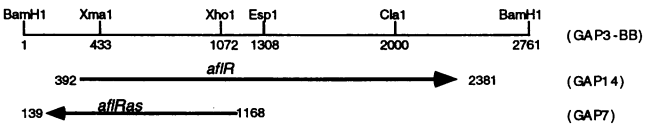

FIG. 1. Schematic representation of the 2.8-kb *Bam*HI fragment that complements the *afl2* mutation of *A. flavus* and its associated cDNAs. The 2.8-kb *Bam*HI fragment (GAP3-BB) is represented by the thin line and its restriction sites. The cDNAs associated with this fragment and their directions of transcription are represented by the arrows. GAP14 was derived from the *aflR* transcript, and GAP7 was derived from the *aflRas* transcript.

RESULTS

We previously isolated a cosmid clone (B9) (27), containing genomic DNA from a wild-type strain (NRRL 3357) of *A. flavus*, that complemented aflatoxin production in *A. flavus* strains carrying the *afl2* mutation (27). The complementing region on the cosmid was localized to a 2.8-kb *Bam*HI fragment of GAP3-BB (Fig. 1). Our initial attempts to identify a transcript from this region were not successful (27). Subsequent screening of a cDNA library (library A) prepared from poly(A)$^+$ RNA collected from cultures during a period of aflatoxin synthesis resulted in the isolation of five clones. The frequency of the clones was rare considering that over 10^6 cDNA plaques were screened. All five clones represented the same transcript, and the three longest were identical in length (1,030 bp). The complete nucleotide sequence of one of the longest clones (GAP7) from library A and its position corresponding to the 2.8-kb *Bam*HI fragment were determined (Fig. 1). The cDNA and genomic sequences of GAP7 were identical, indicating no apparent introns. No similar DNA or peptide sequences were found in the data banks to match the gene sequence of GAP7 or the peptides encoded by the four putative open reading frames ranging from 38 to 83 amino acids. The gene coding for the transcript from which this cDNA was derived is designated *aflRas* in Fig. 1.

Because we were unable to detect a transcript by Northern analysis of RNA isolated from cultures grown as described for library A, we experimented with another method of inducing aflatoxin biosynthesis in cultures of *A. flavus*. We measured the accumulation of aflatoxin in petri dish cultures after resuspension of the mat on either PMS-G, a medium conducive to aflatoxin production, or PMS, a medium that does not induce aflatoxin biosynthesis. Aflatoxin was first detected in the culture medium at 18 h, as shown in Fig. 2. The concentration of aflatoxin increased until 36 h, and then the concentration decreased. This pattern of aflatoxin accumulation was consistently observed in repeated experiments and was similar to the profile of aflatoxin accumulation observed in other resuspension protocols (27). Poly(A)$^+$ RNA was isolated from both induced and noninduced 18-h cultures (Fig. 2), and a Northern blot of the isolated poly(A)$^+$ RNA was hybridized with the 2.8-kb *Bam*HI fragment derived from GAP3-BB (Fig. 1). As shown in Fig. 3, a single band of hybridization was detected on the blot of poly(A)$^+$ RNA from the induced cultures but not on the blot of poly(A)$^+$ RNA from noninduced cultures. The molecular size of the induced transcript was estimated to be 2.0 kb. A cDNA library (library B) was constructed with the poly(A)$^+$ RNA, and four clones corresponding to the 2.8-kb *Bam*HI fragment of GAP3-BB were isolated. Sequence anal-

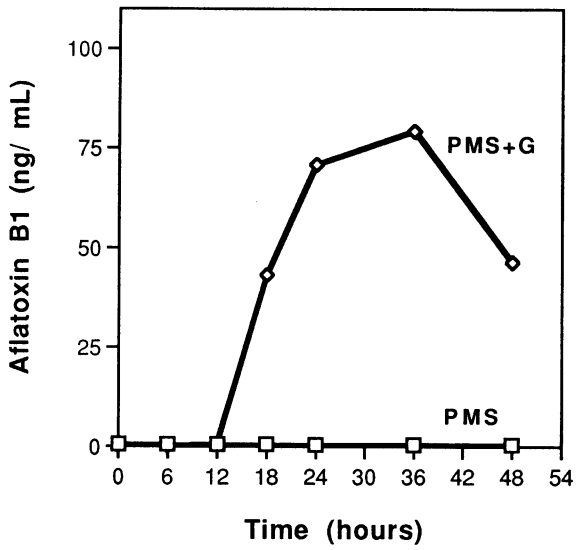

FIG. 2. Aflatoxin B1 accumulation in cultures of wild-type *A. flavus* NRRL 3357 grown by the petri plate method. The fungus was grown in PMS medium in petri plates for 3 days at 28°C and resuspended in PMS or PMS-G and grown at 28°C. Culture filtrate was sampled at 6-h intervals and assayed for aflatoxin B1.

ysis of the cDNAs isolated from library B indicated that these clones represented the same RNA transcript and all were transcribed in the opposite direction from the cDNAs from library A. The position of GAP14, the longest clone from library B, relative to the 2.8-kb *Bam*HI fragment is shown in Fig. 1, and its nucleotide sequence is shown in Fig. 4. The cDNA sequence was identical to that of the genomic DNA, indicating that no introns occur over the transcribed region. Primer extension analysis showed the transcript of *aflR* to be 2,078 bp, 88 nucleotides longer than GAP14 (Fig. 1). An open reading frame was detected starting 25 nucleotides from the 5′ end of GAP14. The open reading frame of 1,311 nucleotides codes for 437 amino acids and a putative protein of 46,679 daltons (Fig. 4). We do not know if there is any significance to the lack of introns in *aflR*, but *aflR* does differ in this respect from the three other genes we have sequenced from *A. flavus* (34, 38, 39); these genes have one to seven short introns. Similarly, the *ver1* and *nor1* genes of *A. parasiticus* have two and three predicted introns, respectively (6, 35). There are no TATA or CAAT boxes in the genomic DNA of *aflR* 5′ to the transcriptional start site. This was not unexpected, as filamentous fungi are quite variable with respect to transcriptional initiation sites (14) and the *benA* gene (34) from *A. flavus* lacks these motifs. In contrast, the *ver1* and *nor1* genes from *A. parasiticus* and the *adh1* gene of *A. flavus* each contain a short sequence characteristic of a TATA box motif (6, 35, 38).

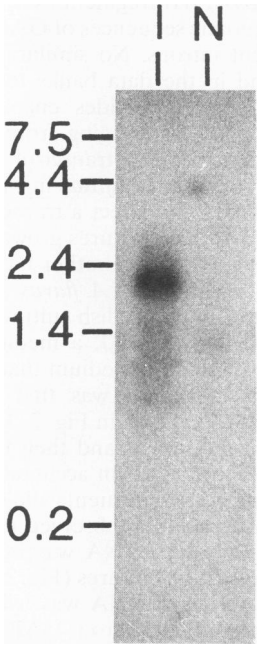

FIG. 3. Northern analysis of *aflR* under induced and noninduced conditions. Poly(A)$^+$ RNA was isolated from wild-type *A. flavus* NRRL 3357 resuspended in either a medium conducive (PMS-G [lane I]) or a medium nonconducive (PMS [lane N]) to aflatoxin production. A 10-μg sample of poly(A)$^+$ RNA was loaded per well, electrophorested, blotted, and hybridized with the 2.8-kb *Bam*HI fragment (GAP3-BB). The numbers on the left are molecular sizes in kilobases.

Analysis of the predicted amino acid sequence indicated that the putative AFLR polypeptide contains a zinc cluster motif between amino acid positions 29 and 56. This region contains the consensus sequence Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys. This motif has been found in several fungal transcriptional regulatory proteins (1, 9, 13, 32). Figure 5 shows the amino acid alignment of the zinc cluster region of the putative *A. flavus* AFLR protein with those reported in several other fungal proteins. Perfect alignment of the cysteine residues was found with proteins GAL4 (16), PPR1 (17), PUT3 (24), and ARGR2 (10) from *Saccharomyces cerevisiae*, MAL63 (19) from *S. carlsbergensis*, LAC9 (30) from *Klyveromyces lactis*, and QA-1F (1) from *Neurospora crassa*. Also conserved in AFLR is the proline residue that has been shown in GAL4 to be necessary for DNA binding and function. Interestingly, these transcriptional activators are similar to AFLR in that they also lack introns.

Previous studies have suggested that *aflR* is involved in the regulation of aflatoxin biosynthesis (4, 27). To determine if other polyketide-producing fungi have genes similar to *aflR*, Southern blots of *Bam*HI-digested genomic DNAs from seven fungal species were hybridized with the *aflR* gene. *A. parasiticus* was chosen because it also produces aflatoxin. *A. oryzae* and *A. sojae* were chosen because they belong to the same fungal taxon (*Aspergillus* section *Flavi*) as *A. flavus* and *A. parasiticus* (20). *A. nidulans* 1079 was chosen because it has been reported to produce sterigmatocystin, one of the metabolites produced late in the aflatoxin pathway. *P. griseofulvum* was chosen because a number of *Penicillium* species are known to produce the polyketide 6-methylsalicylic acid. The fungi *C. heterostrophus* and *C. kikuchii* were chosen because they each produce polyketide phytotoxins. As shown in Fig. 6, DNAs from species closely related to *A. flavus* (*A. parasiticus*, *A. oryzae*, and *A. sojae*) hybridized to the radiolabeled 2.8-kb *Bam*HI fragment of GAP3-BB (Fig. 1). DNAs from *A. nidulans* and fungi of the other genera did not. In each of the *A. flavus* strains, the probe specifically hybridized to a single 2.8-kb fragment. The hybridization with genomic DNAs from two *A. parasiticus* strains yielded the same hybridization pattern but a pattern distinct from that of *A. flavus*. The hybridization profile of *A. parasiticus* showed one major band (1.8 kb) and two minor bands (1.3 and 0.7 kb) of hybridization. The similarity of the hybridization patterns among strains within the species suggests that the genetic organization around *aflR*

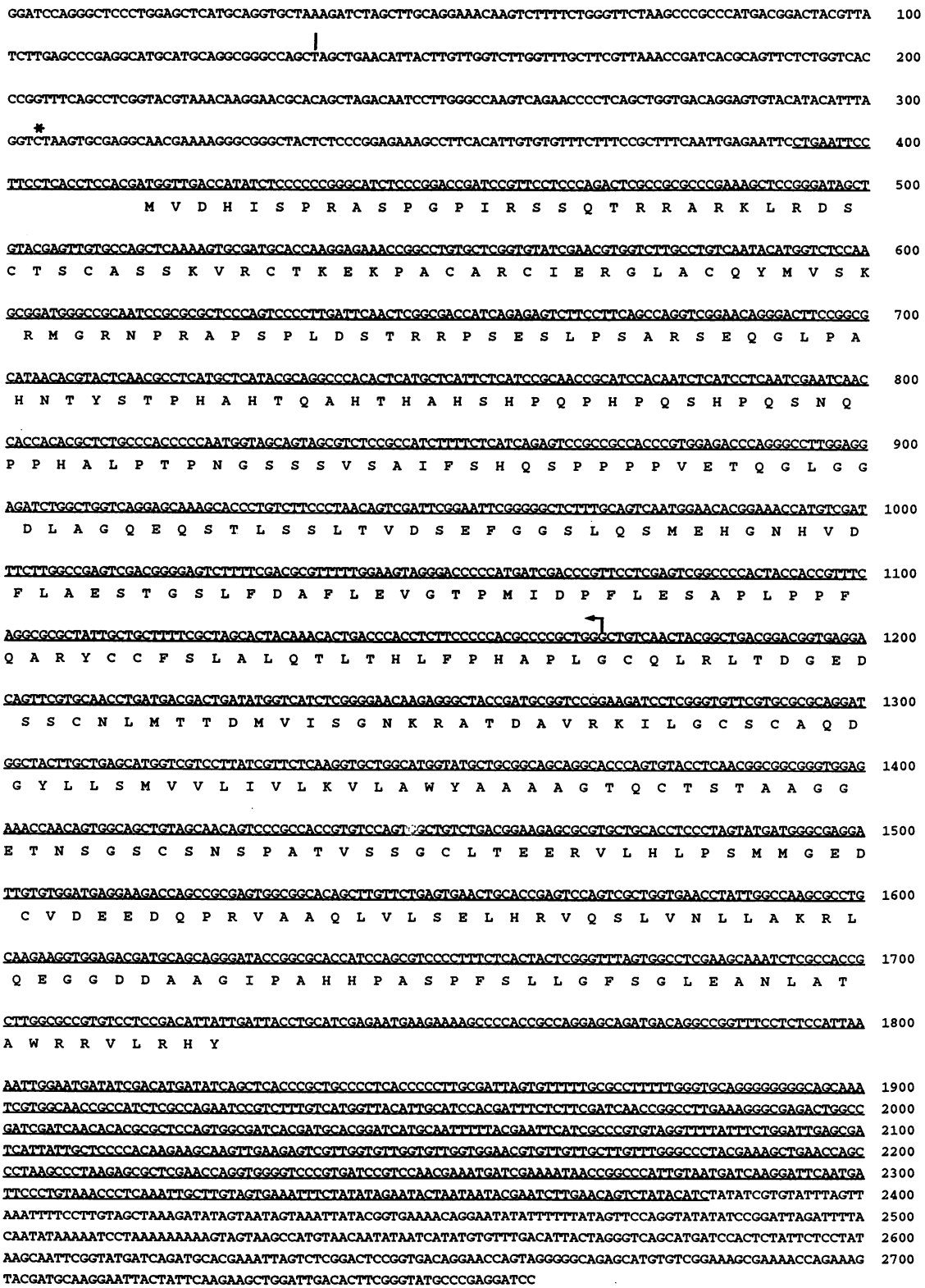

FIG. 4. DNA sequence of the 2.8-kb *Bam*HI fragment that complements the *afl2* mutation of *A. flavus*. The underlined sequence represents the *aflR* cDNA (GAP14) derived from cDNA library B. The letters below the nucleotide sequence represent the putative amino acid sequence of AFLR, a 46-kDa polypeptide containing a zinc cluster motif. The asterisk represents the transcriptional start site of *aflR* determined by primer extension analysis. The 5′ end of the 1,030-bp *aflRas* cDNA (GAP7) derived from cDNA library A is represented by the arrow, and its 3′ terminus is represented by the vertical bar.

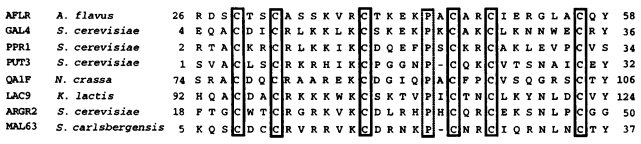

FIG. 5. Amino acid sequence alignment of the zinc cluster motif of the predicted AFLR protein and seven other transcriptional activator proteins. Conserved cystiene residues are indicated by the solid boxes, and the conserved proline residue is indicated by the broken box. The numbers preceding and following each sequence refer to the amino acid positions of the respective proteins. Hyphens represent gaps introduced into the sequence for better alignment.

is conserved. Genomic DNA fragments from *A. sojae* and *A. oryzae*, two important fermentation species, also hybridized to the *aflR* probe. The hybridization patterns of *A. oryzae* and *A. sojae* were identical to each other and similar to that of *A. parasiticus*. These two fungal species had 1.8- and 0.7-kb bands of hybridization.

DISCUSSION

Biochemical evidence from our previous work with *afl2* (27) and *apa2* (4) suggested that the products of these genes regulate aflatoxin biosynthesis in *A. flavus* and *A. parasiticus*. Furthermore, we have shown that *afl2* is required for expression of the *nor1* and *ver1* genes in *A. flavus* (39). The presence of nucleotide sequences coding for a putative protein containing a zinc cluster motif in both of these fungi further supports the hypothesis that the products of these two genes are involved in regulation of the aflatoxin pathway. Complete sequence analysis of the genomic and cDNA clones indicated that the two genes are 96% similar at the nucleotide level and 97% similar at the amino acid level. We also found that *apa2* complements aflatoxin production in *A. flavus* 656-2 (27), which has a defective *afl2* gene (39). Thus, our data indicate that *afl2* and *apa2* are homologs of the same gene. In keeping with the convention of nomenclature for *Aspergillus* genes (8), we suggest that these genes be given the same name. We propose that the gene be designated *aflR* because several lines of evidence suggest that it is a regulatory gene. As will be discussed later, we propose that the DNA region containing *aflR* be referred to as a locus, as we have evidence that there are two transcripts from this locus.

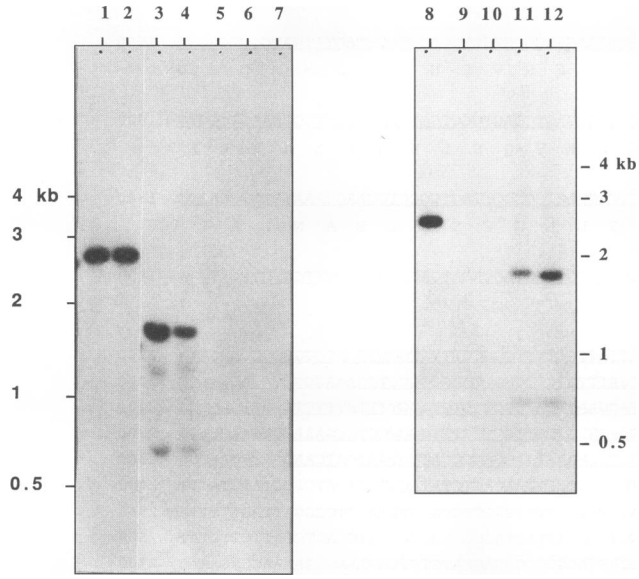

FIG. 6. Presence of *aflR* in other fungal species. Fungal genomic DNA was digested with *Bam*HI, electrophoresed, blotted, and hybridized with the 2.8-kb *Bam*HI fragment of GAP3-BB. Lanes: 1, *A. flavus* NRRL 3357; 2, *A. flavus* 650-33; 3, *A. parasiticus* SU-1; 4, *A. parasiticus* 2999; 5, *A. nidulans* 1079; 6, *C. kikuchii*; 7, *C. heterostrophus*; 8, *A. flavus* 650-33; 9, *P. griseofulvum* 1952; 10, *P. griseofulvum* 994; 11, *A. oryzae* 3483; 12, *A. sojae* 5594.

The zinc cluster motif in the putative AFLR protein is similar to a group of fungal transcriptional activators associated with a number of metabolic pathways that contain six cysteines with identical spacing: Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys. Best known of this group of transcription activators is GAL4 from *S. cerevisiae*, which regulates transcription of the genes involved in galactose metabolism. Recent crystallographic findings on GAL4 indicate that this motif binds two molecules of zinc, forming a zinc cluster configuration involving all six cysteines (25). The conformational structure formed by the cluster is thought to be a component of the DNA-binding domain. Furthermore, these studies have indicated that GAL4 binds as a homodimer to its target genes. The target sequence for several of the zinc cluster proteins (GAL4, PUT3, and PPR1) are two rotationally symmetric, 3-bp sites (CGG...GCC) separated by 11, 16, and 6 bp, respectively. By using chimeras of these three proteins, Reece and Ptashne (29) have shown that binding to the nucleotide triplets is not specified by the zinc cluster motif but rather by a region 19 amino acids to the carboxy-terminal side of the zinc cluster. This region comprises the linker and the N-terminal amino acid sequence of the dimerization elements. It is suggested that this region on the protein specifically positions the zinc cluster to facilitate binding.

Two genes that AFLR is hypothesized to regulate are *nor1* and *ver1*. Examination of the promoter regions of these genes revealed CGG...GCC motifs similar to those found in the promoters of the target genes of GAL4, PUT3, and PPRI. However, the number of base pairs separating the conserved sites was not consistent among the motifs within a promoter, nor was any single motif conserved between the promoters. We also examined the putative amino acid conformation of AFLR on the carboxy-terminal side of the zinc cluster. There was no apparent predicted alpha-helical secondary structure that is typical of the proteins that bind as dimers. However, other zinc cluster DNA-binding proteins, such as ARGR2 and MAL63, also lack the coiled-coil dimerization element and are thought to bind as monomers or possibly in combination with other proteins (29). The target binding site for the ARGR2 protein has a single CG^{G}_{A} motif. Several of these target sequences are present in the promoters of the *nor1* and *ver1* genes. Whether any of these motifs are actually AFLR-binding regions is the focus of our current research. Gel retardation studies will determine if AFLR binds to the upstream nucleotide sequences of the *nor1* and *ver1* genes of *A. flavus*.

We also present evidence in this study that *aflR* codes for two overlapping transcripts that are derived from opposite DNA strands. In addition to the gene (*aflR*) that codes for the zinc cluster DNA-binding motif, we isolated a cDNA derived from the opposite DNA strand. We have designated the gene coding for this transcript *aflRas*. Although we do not know the function of the *aflRas* transcript in *A. flavus*, there is evidence in the literature to support in situ gene regulation of eukaryotes by antisense RNA (15, 22). In the fungus *Dictyostelium discoideum*, antisense RNA functions to regulate the

presporulation gene *EB4*, possibly by destabilizing the sense transcript. Transcripts of *EB4* accumulate during the aggregation stage of *D. discoideum* development but are not detectable during the vegetative stage. Studies have shown that *EB4* is constitutively transcribed, and the transcription of the antisense RNA is five times higher in the vegetative stage than in the aggregation stage. Through a series of experiments, Hildebrandt and Nellen showed that the accumulation of antisense transcripts destabilizes the mRNA of *EB4* (15). We have no evidence that the *aflRas* transcript in *A. flavus* functions in an analogous mechanism; however, it is interesting that the 3′ region of the *aflRas* strand overlaps the promoter region of the *aflR* transcript, as is the case with the two transcripts of *EB4*. It is also interesting that we were not able to detect accumulation of both the sense and antisense transcripts in the same cDNA libraries, suggesting that induction of these two species may be dependent on culture conditions. In some organisms, the antisense message has been shown to be unstable. Our failure to detect these antisense transcripts on Northern blots may be due to instability of the message. Overlapping transcripts also have been found from the *Bz2* locus in *Zea mays* (33). In this case, the antisense strand had no open reading frame and was found only when the sense strand was produced in abundance. Although such a situation at first seems to negate a regulatory role for the antisense strand, the investigators argued that the antisense strand may be produced in response to high expression of the sense strand to down-regulate the expression of the sense strand. More work is needed to establish a role for the *aflRas* transcript and to show that it is not an artifact of our cDNA cloning.

Because several filamentous fungi produce polyketides, we examined a number of fungal species, including those closely related to *A. flavus*, for the presence of genes similar to *aflR*. The presence of genes similar to *aflR* in *A. oryzae* and *A. sojae* is not unexpected, as both of these fungi show a high degree of DNA relatedness. Kurtzman et al. (21) reported that *A. flavus* and *A. oryzae* have 100% DNA complementarity and *A. parasiticus* and *A. sojae* have 91% complementarity. *A. flavus* and *A. parasiticus*, by comparison, showed 70% complementarity. They argued that their data supported the concept that these four species represent morphological and physiological variants of a single species (20). Given the small number of strains hybridized in this study, it is not possible to draw conclusions about the genetic similarity of these strains on the basis of their hybridization with the *aflR* gene. However, the hybridization patterns suggest that at this locus, *A. oryzae* is more related to *A. parasiticus* than to *A. flavus*.

The presence of genes with similarity to *aflR* within the *A. oryzae* and *A. sojae* genomes raises an interesting question about the function of these genes. Neither of these two species produces aflatoxin, and there is no report of the presence of either pathway genes or pathway metabolites in them. Because these species are very closely related to *A. flavus* and *A. parasiticus*, one may expect them to have some of the genes of the aflatoxin pathway. Additional studies on the molecular biology of these species are needed to address this question.

The *aflR* gene did not hybridize with genomic DNA from *P. griseofulvum*, *A. nidulans*, *C. kikuchii*, or *C. heterostrophus* under stringent conditions. We expected hybridization with DNA of *A. nidulans* 1079, since this strain has been reported to produce sterigmatocystin, one of the metabolites produced late in the aflatoxin biosynthetic pathway (18). These data suggest that if *A. nidulans* has a gene similar to *aflR*, the sequence of the gene has diverged enough from the sequences of its counterpart genes in the fungi of *Aspergillus* section *Flavi* that it could not be detected under the stringency used in our studies.

Understanding the molecular regulation of aflatoxin biosynthesis is a long-term goal of our laboratory. Data presented in this report and others have indicated that *aflR* is a regulatory gene. Because the *aflR* gene appears to play a pivotal role in the aflatoxin biosynthetic pathway, studies on the regulation of *aflR* itself and its regulation of other pathway genes may allow the development of novel strategies for controlling aflatoxin contamination.

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