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Isolation and Characterization of a Gene from *Aspergillus parasiticus* Associated with the Conversion of Versicolorin A to Sterigmatocystin in Aflatoxin Biosynthesis

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DNA isolated from the wild-type aflatoxin-producing (Afl$^+$) fungus *Aspergillus parasiticus* NRRL 5862 was used to construct a cosmid genomic DNA library employing the homologous gene (*pyrG*) encoding orotidine monophosphate decarboxylase for selection of fungal transformants. The cosmid library was transformed into an Afl$^-$ mutant, *A. parasiticus* CS10 (*ver-1 wh-1 pyrG*), deficient in the conversion of the aflatoxin biosynthetic intermediate versicolorin A to sterigmatocystin. One *pyrG*$^+$ Afl$^+$ transformant was identified. DNA fragments from this transformant, recovered by marker rescue, contained part of the cosmid vector including the *pyrG* gene, the *amp*^{r} gene, and a piece of the original genomic insert DNA. Transformation of these rescued DNA fragments into *A. parasiticus* CS10 resulted in production of wild-type levels of aflatoxin and abundant formation of sclerotia. The gene responsible for this complementation (*ver-1*) was identified by Northern RNA analysis and transformation with subcloned DNA fragments. The approximate locations of transcription initiation and polyadenylation sites of *ver-1* were determined by an RNase protection assay and cDNA sequence analysis. The predicted amino acid sequence, deduced from the *ver-1* genomic and cDNA nucleotide sequences, was compared with the EMBL and GenBank data bases. The search revealed striking similarity with *Streptomyces* ketoreductases involved in polyketide biosynthesis.

Aflatoxins are low-molecular-weight secondary metabolites that are produced by the imperfect fungi *Aspergillus flavus* and *A. parasiticus*. These compounds are known to be potent animal hepatocarcinogens and are suspected to be carcinogens of humans (14). Frequent aflatoxin contamination of agricultural commodities (39) presents a potential threat to the health of animals and humans. The foremost objective of our research is the eventual elimination of aflatoxin contamination in food and feed. The biosynthesis of aflatoxins is believed to occur through the condensation of acetate subunits by a polyketide synthetase pathway to form the anthraquinone norsolorinic acid (NA). NA is the earliest stable biosynthetic pathway intermediate that has been identified. The remaining steps in the putative biosynthetic scheme for the conversion of NA to the xanthone sterigmatocystin (ST) and eventually to aflatoxin B_1 are as follows: NA → averantin (AVN) → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A (VA) → sterigmatocystin (ST) → *O*-methylsterigmatocystin → aflatoxin B_1 (9, 27).

Currently, there is a limited understanding of the details of the molecular mechanisms that regulate aflatoxin production. Investigation into the regulation of this biosynthetic pathway at the molecular level has proceeded primarily by three different strategies: (i) identification and purification of proteins involved in conversion steps leading to aflatoxins, (ii) differential hybridization analysis for isolation of genes involved in aflatoxin biosynthesis, and (iii) complementation analysis with a genomic DNA library to transform fungal mutants deficient in aflatoxin biosynthesis. A versiconal cyclase involved in dehydration of versiconal (43), a NA dehydrogenase (8, 16) that is associated with the conversion of NA to averantin, and a methyltransferase (10) that catalyzes the conversion of ST to *O*-methylsterigmatocystin were only recently purified to homogeneity. Several other enzyme activities in the biosynthetic scheme have also been described (2, 3, 17, 18, 38, 41, 58–60). Using genetic complementation of mutant strains blocked in aflatoxin biosynthesis, Payne et al. (50) successfully isolated an *A. flavus* DNA fragment that appears to carry a gene related to aflatoxin biosynthesis.

In our laboratory we have focused primarily on the isolation of *A. parasiticus* genes that are involved in specific aflatoxin biosynthetic conversion steps. The isolation of aflatoxin-associated genes was accomplished by genetic complementation of *A. parasiticus* mutants (*ver-1* and *nor-1*) (37, 56) that are blocked at unique conversion steps in the proposed pathway by using a cosmid genomic DNA library from a toxigenic *A. parasiticus* strain. This approach has allowed us to clone the *nor-1* (previously called *nar-1*, for NA related) gene, which encodes an activity associated with the bioconversion of NA to AVN (13), and the *ver-1* gene (55), which is associated with the conversion of VA to ST (this study). These newly isolated genes will be used as probes to study regulation of gene expression during aflatoxin biosynthesis. They will also be used for the development of genetically stable atoxigenic fungal strains by methods of gene disruption, which can then be used for competitive exclusion of aflatoxin-producing strains in the field. Biocompetition of this type has proven to be quite successful in laboratory and field tests (19, 22, 23, 28). Additionally, an understanding of the control mechanisms involved in aflatoxin biosynthesis may lead to the development of agents or genetically engineered plants that inhibit toxin production.

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MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* HB101 [*hsdS20* (r_B^- m_b^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* Sm^r *xyl-5 mtl-1 supE44*] was used for propagating plasmids. *E. coli* LE392 [F^- e14$^-$ (*mcrA*) *hsdR514* (r_k^- m_k^+) *supE44 supF58 lacY1 galK2 galT22 metB1 tryR55*] was the recipient strain for packaged lambda phage. *A. parasiticus* NRRL 5862 (SU-1) served as the aflatoxin-producing wild-type strain. *A. parasiticus* CS10 (*ver-1 wh-1 pyrG*) (56), derived from *A. parasiticus* ATCC 36537 (*ver-1 wh-1*) (5), was used in complementation studies for the isolation of the *ver-1* gene. *A. parasiticus* ATCC 36537 was obtained by UV mutagenesis of *A. parasiticus* NRRL 5862. This blocked mutant is unable to convert VA to ST. Although the exact nature of the *ver-1* mutation is not known, neither *A. parasiticus* ATCC 36537 (5, 9) nor *A. parasiticus* CS10 produces detectable levels of aflatoxins in liquid or solid growth media. Additionally, neither strain has been observed or reported to revert back to Afl^+.

Fungal strains were maintained on potato dextrose agar (PDA) or Czapek-Dox agar (CZA) that served as a defined medium. Coconut agar medium (CAM) was used for screening fungal strains for aflatoxin accumulation by visualization of blue fluorescence under UV light (24). Cultures were incubated at 32°C unless otherwise indicated.

Chemicals. Chemicals, unless specifically referenced in the text, were purchased from Sigma Chemical Co. PDA and CZA were purchased from Difco Laboratories (Detroit, Mich.).

Transformation of *A. parasiticus* CS10. Protoplasts were transformed with polyethylene glycol as previously described (56). Cotransformations were occasionally used to transform *A. parasiticus* CS10 to *pyrG*$^+$. Under these conditions, the *pyrG* selectable marker was included on a plasmid (pBP28) that was separate from the vector containing the transforming DNA of interest. Plasmid pBP28 was constructed by replacing the 0.28-kb *Bam*HI-*Sal*I fragment from pBR322 with a 2.8-kb *Bam*HI-*Sal*I DNA restriction fragment containing the *pyrG* gene from pPG3J. The vector containing the DNA of interest was added in a twofold molar ratio over pBP28 for a total of ≈3 μg of DNA in the transformation mixture.

Isolation and analysis of genomic DNA from fungal cells. Genomic DNA was isolated from fungal mycelium by using a phenol-chloroform protocol developed for mammalian DNA (4). Restriction endonuclease enzymes were purchased from Bethesda Research Laboratories, Inc., and used according to the supplier's instructions. Southern hybridization analyses (46) were performed with ^{32}P-labelled DNA probes generated by the random primer procedure (random-primed DNA labeling kit; Boehringer Mannheim Biochemicals) as previously described (56).

Preparation of cosmid library. The cosmid vector pBZ5 (Fig. 1) was constructed by ligation of the 2.8-kb *Bam*HI-*Sal*I *pyrG*-containing restriction fragment from plasmid pPG3J into the *Hin*dIII restriction endonuclease site of the cosmid vector pHC79 (Stratagene, La Jolla, Calif.). Ligation of the blunt-ended *pyrG* fragment in pBZ5 regenerated the *Hin*dIII site, since end filling of the *Sal*I and *Hin*dIII restriction endonuclease sites completed the recognition sequence for *Hin*dIII. The ability to transform *A. parasiticus* CS10 to prototrophy at efficiencies comparable to that obtained with pPG3J confirmed the presence of the *pyrG* gene in pBZ5.

The cosmid genomic DNA library was prepared by the procedures of Maniatis et al. (46). DNA from the wild-type

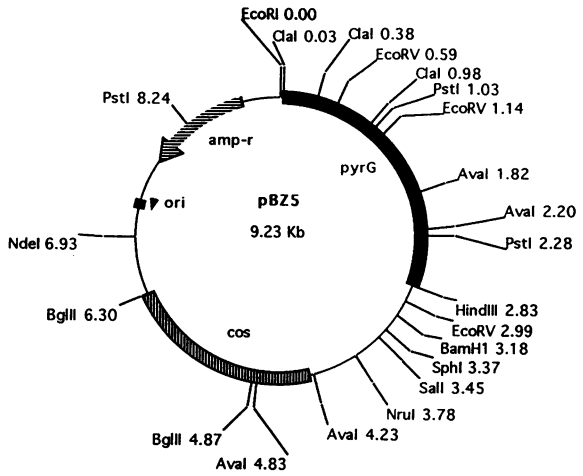

FIG. 1. Cosmid vector pBZ5 used in the preparation of an *A. parasiticus* NRRL 5862 cosmid genomic DNA library. This vector was constructed by blunt end ligation of a 2.8-kb *Bam*HI-*Sal*I *pyrG*-containing fragment from plasmid pPG3J (56) into the *Hin*dIII restriction endonuclease site of cosmid pHC79. DNA from a toxigenic *A. parasiticus* strain was digested with *Sau*3A, size fractionated, and then ligated into the alkaline phosphatase-treated *Bam*HI site of pBZ5.

Afl^+ strain *A. parasiticus* NRRL 5862 was used for library preparation. This DNA was partially digested by the restriction endonuclease *Sau*3A and size fractionated by sucrose density gradient centrifugation. Fractions containing DNA fragments between 30 and 40 kb in size were ligated into the alkaline phosphatase-treated *Bam*HI site of pBZ5. Concatemers were then packaged into lambda phage particles with a commercial packaging extract (Promega Corp., Madison, Wis.).

The DNA library was completed by transfecting *E. coli* LE392 with recombinant phage particles and transferring these cells to LB agar medium containing ampicillin (50 μg/ml) (46). Overnight incubation at 37°C yielded approximately 870 CFU per petri plate (11 by 150 mm). Suspensions of cells harvested from 10 plates were used for cosmid isolation by alkaline lysis and CsCl purification (46).

Preparation of cDNA library. *A. parasiticus* NRRL 5862 was inoculated into a glucose-mineral salts medium (1) and grown for 48 h. RNA isolated from the mycelium by hot phenol purification (4) was then used to prepare a cDNA library. This library was constructed by Stratagene with their Uni-Zap XR vector.

Screening of the cosmid genomic DNA library by genetic complementation of *A. parasiticus* CS10. DNA from the combined cosmid DNA library was used in transformation of *A. parasiticus* CS10. Protoplasts were plated directly onto CAM supplemented with 20% sucrose as an osmotic stabilizer. CAM does not contain sufficient uridine to support the growth of untransformed protoplasts. Only protoplasts receiving the *pyrG* marker, contained on the cosmid, were capable of growth on CAM. Transformant colonies displaying blue fluorescence under longwave UV light were isolated and reinoculated onto fresh PDA for further analysis and verification of aflatoxin production.

Confirmation of aflatoxin production. Conidia (10^7) from transformant colonies were inoculated into 20 ml of YES (2% yeast extract, 20% sucrose [pH 5.5]) medium contained in a 50-ml Erlenmeyer flask. Each flask also contained four

4.5-mm glass beads to aid in aeration of the culture and to facilitate dispersed mycelial growth (33, 52). Cultures were then grown for 3 days at 28°C in an orbital shaker (150 rpm).

After incubation, cultures were filtered through miracloth, and the filtrate was used for identification of aflatoxins. Only the filtrate was analyzed for aflatoxins, since aflatoxins B_1 and G_1 are known to pass efficiently through the mycelial walls into the surrounding medium (44). Filtrates from cultures of *A. parasiticus* CS10 and *A. parasiticus* NRRL 5862 were included as aflatoxin production-negative and -positive controls, respectively. Filtrates were tested by using thin-layer chromatography (TLC) and an enzyme-linked immunosorbent assay (ELISA).

TLC analyses were performed on activated high-performance silica TLC plates (10 by 10 cm) in an equilibrated chamber with chloroform-methanol (97:3) as a solvent system. VA (kindly supplied by L. Lee and D. Bhatnagar; U.S. Department of Agriculture-Agricultural Research Service, New Orleans, La.) and aflatoxins B_1, B_2, G_1, and G_2 were resolved on each plate as reference standards. Direct competitive ELISA analyses were performed as described by Pestka (51) with aflatoxin B_1 monoclonal antibodies (kindly provided by J. Pestka, Michigan State University) and aflatoxin B_1-horseradish peroxidase conjugate prepared by the method of Chu et al. (15).

Sclerotium production. Transformant isolates were tested for their ability to produce sclerotia. *A. parasiticus* NRRL 5862 and *A. parasiticus* ATCC 36537 were included for comparisons. Approximately 10^3 previously frozen conidia (in 15% glycerol) of each strain were inoculated on the center of a petri plate (100 by 15 mm) prepared with 25 ml of PDA medium. Cultures were incubated at 30 and 37°C for 10 days. Sclerotia were harvested as described by Cotty (20), counted, and then dried at 50°C for 48 h. Sclerotial diameters were measured with the Java video analysis system (Jandal Corp., Corte Madera, Calif.). Between 32 and 100 sclerotia were chosen at random from each plate and measured from top to bottom near the estimated center of the vertical plane.

Marker rescue of the *amp*^{r} gene from an aflatoxin-producing (Afl$^+$) transformant. Four restriction endonucleases (*Sal*I, *Sph*I, *Bgl*II, and *Nde*I) were used to digest genomic DNA from one *A. parasiticus* CS10 Afl$^+$ transformant clone. These enzymes were chosen because they cut once within the cosmid vector pBZ5 but do not cut within the *pyrG* or *amp*^{r} gene. The strategy was designed to allow recovery of fragments that contained the *pyrG* gene, the *amp*^{r} gene, and presumably a piece of the original *A. parasiticus* insert DNA because the second restriction endonuclease site must be situated somewhere within the flanking DNA.

Each restriction enzyme was used separately to digest 50 μg of DNA from the Afl$^+$ transformant. DNA was separated on a 1.2% agarose gel. Regions of the gel corresponding to the desired fragments containing the *amp*^{r} gene were removed and used for DNA isolation with Prep-A-Gene DNA isolation matrix (Bio-Rad, Richmond, Calif.). Isolated DNA was diluted (2 ng of DNA per μl) before T4 DNA ligase treatment to minimize intermolecular ligation. Circularized fragments were transformed into *E. coli* DH5α, which was then grown on LB agar medium containing ampicillin (50 μg/ml).

Isolation and analysis of RNA from fungal cells. Conidia (10^7) of *A. parasiticus* were inoculated into 100 ml of YES broth and grown at 28°C in an orbital shaker (150 rpm) for 3 days. The resulting mycelium was filtered through Miracloth and then quickly frozen in liquid nitrogen. RNA was then isolated by hot phenol purification (4). Polyadenylated RNA was isolated by affinity column chromatography with an oligo(dT) matrix (4). Northern analysis of RNA samples was performed as described by Maniatis et al. (46).

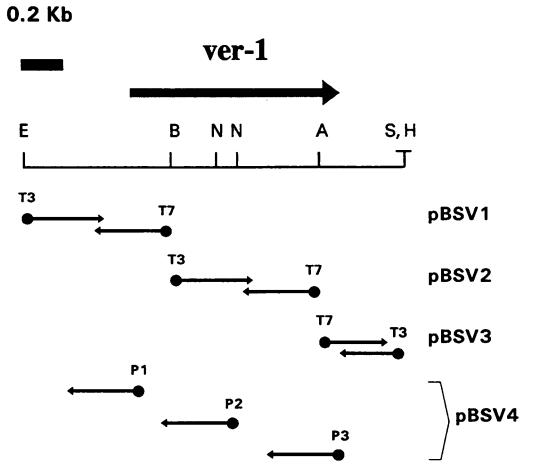

FIG. 2. Restriction endonuclease map and sequencing strategy of the 1.8-kb *Eco*RI-*Hin*dIII fragment. The shaded arrow indicates the direction of transcription for the *A. parasiticus ver-1* gene. Regions of DNA cloned into pBluescriptII plasmids for the construction of pBSV1, pBSV2, and pBSV3 are illustrated directly beneath the corresponding areas of the restriction map. The orientations of T7 and T3 primer regions, contained on pBluescriptII plasmids, are included with arrows designating the directions and extents of sequencing. Each arrow may represent data from more than one set of sequencing reactions. Oligonucleotide primers (P1, P2, and P3) were synthesized and used to sequence plasmid pBSV4, which contains the 1.8-kb *Eco*RI-*Sal*I fragment cloned into pBluescriptII KS−. E, *Eco*RI; B, *Bam*HI; N, *Nde*I; A, *Ava*I; S, *Sal*I; H, *Hin*dIII.

Analysis of RNA transcripts from the *ver-1* gene. The orientation of the *ver-1* transcript was first determined by Northern hybridization analysis with strand-specific riboprobes made from plasmid pBSV1 (Fig. 2), in which a 0.7-kb *Eco*RI-*Bam*HI *ver-1* fragment was ligated into the plasmid pBluescriptII KS− (Stratagene). Bluescript plasmids contain T7 and T3 phage promoters on either side of the polylinker region that allow transcription of DNA inserts in both directions. Plasmid pBSV1 was linearized with restriction endonuclease enzymes *Sac*I and *Sal*I (located in the polylinker) to generate T3 and T7 runoff transcripts, respectively. Radiolabelled riboprobes were made according to the manufacturer's instructions.

The 5′ and 3′ ends of the *ver-1* transcript were localized by using an RNase protection assay (4). Riboprobes for 5′-end analysis were transcribed with T7 polymerase with *Sal*I-linearized plasmid pBSV1 (Fig. 2). Riboprobes for 3′-end analysis were prepared with T7 polymerase and *Eco*RI-linearized plasmid pBSV3 (Fig. 2), in which a 0.4-kb *Ava*I-*Hin*dIII *ver-1* fragment was blunt end ligated into the *Sma*I site of pBluescriptII SK−. Riboprobes were hybridized at 55°C to mRNA from *A. parasiticus* NRRL 5862 or yeast trRNA (control). Samples were treated with RNase T1 and RNase A and separated by electrophoresis on a 5% polyacrylamide gel under denaturing conditions. Acrylamide gels were dried under vacuum and subjected to autoradiography with Kodak XAR5 film.

Sequence analysis. Nucleotide sequence analysis of genomic DNA was performed by the dideoxy-chain termination method (53) with Sequenase II (U.S. Biochemical

Corp., Cleveland, Ohio) according to the manufacturer's instructions. The DNA inserts in plasmids pBSV1, pBSV2, and pBSV3 were sequenced on both strands by using the T7 and T3 primers supplied in the kit (Fig. 2). Plasmid pBSV2 was constructed by ligation of a blunt-ended 0.6-kb *Bam*HI-*Ava*I internal *ver-1* fragment into the *Sma*I site of pBluescriptII SK−. From this initial sequence information, oligonucleotide primers (15 nucleotides) were synthesized at the Michigan State University Molecular Structure Facility and used to confirm the sequence and to remove ambiguities. Plasmid pBSV4 (Fig. 2), which contains a 1.8-kb *Eco*RI-*Sal*I fragment, served as a template strand for sequencing with the synthesized oligonucleotide primers.

Sequence analysis of the *ver-1* cDNA fragment was performed with a TAQuence sequencing kit (U.S. Biochemical Corp.), which utilizes the thermostable Δ*Taq* DNA polymerase. The cDNA fragment was isolated by in situ plaque hybridization of the cDNA library with plasmid pBSV2, which contains an internal portion of the *ver-1* gene.

Computer analysis of sequence data. Computer analyses of nucleotide data were performed by using the Wisconsin Genetics Computer Group package. The location of open reading frames, a translation start codon, and introns were predicted by using the software programs Frames, Testcode, and codon preference. Codon usage files were constructed by using data from 45 different *A. nidulans* genes reported by Lloyd and Sharp (45). Amino acid comparisons were made with a window size of 30 and a stringency of 15.

Nucleotide sequence accession number. The GenBank accession number for *A. parasiticus ver-1* is M91369.

RESULTS

Library preparation. DNA purified from *A. parasiticus* NRRL 5862 was confirmed by field-inversion gel electrophoresis to be of high molecular weight (100 to 150 kb). The efficiency of packaging for the ligated concatamers was deduced by estimating the ability of phage particles to transfect *E. coli* LE392 (1.65×10^5 transfectants per μg of DNA). Cosmid preparations from 12 randomly isolated *E. coli* transfectant clones revealed the average size of the recombinant cosmid to be approximately 45 kb. Therefore, the average size of *A. parasiticus* DNA inserted into the original cosmid pBZ5 (9.2 kb) was estimated to be 36 kb. Approximately 2,200 clones of this size would provide a 95% probability of obtaining a region of interest if one assumes that the total genome size of *A. parasiticus* is similar to that of *A. nidulans* (2.6×10^4 kb) (57).

Transformation of *A. parasiticus* CS10 with a pooled DNA library. *A. parasiticus* CS10 was transformed with the combined cosmid DNA library at an efficiency of 29 *pyrG*$^+$ transformants per μg of DNA. Approximately 400 CFU were visible after 2 days of incubation at 32°C. No growth was observed on the control plates inoculated with protoplasts that were not treated with DNA. The morphologies of almost all of the transformant colonies were the same as that of the parent strain, *A. parasiticus* ATCC 36537, grown under similar conditions. One colony fluoresced blue under longwave UV light on day 4 of incubation. Reinoculation of this putative Afl$^+$ isolate onto CAM demonstrated that it still retained its ability to produce a blue-fluorescing compound, which was released into the surrounding medium. Additionally, it no longer accumulated significant quantities of the bright yellow fluorescent pigment seen in the recipient strain, which contains VA as a major component (42).

Marker rescue of *amp*^{r} gene. Southern analysis of DNA purified from the putative Afl$^+$ transformant with the cosmid vector pHC79 as a probe suggested that 12.5-kb *Sal*I, 9.8-kb *Bgl*II, 11.0-kb *Sph*I, and 7.2-kb *Nde*I restriction fragments contained the *amp*^{r} and *pyrG* genes plus a piece of the original *A. parasiticus* DNA insert. Ampicillin-resistant clones containing plasmids made by circularization of each of these restriction endonuclease fragments were isolated. Restriction endonuclease analysis of each rescued plasmid with the original restriction enzyme used for its isolation confirmed that plasmid sizes were consistent with predictions based on Southern analysis of DNA from the Afl$^+$ transformant (Fig. 3). Isolated plasmids were designated pVC1A (*Bgl*II), pVC1B (*Nde*I), pVC1C (*Sal*I), and pVC1D (*Sph*I) for the restriction endonuclease enzymes used in their recovery. All plasmids appeared to have overlapping restriction endonuclease sites, and most of the DNA from cosmid pBZ5, except for a deletion of approximately 2 kb in the region of the lambda cohesive site, was apparently present. It is possible that only part of the cosmid integrated or that rearrangement of the cosmid occurred during transformation. Additionally, deletions in cosmids of this size are quite common during replication in *E. coli* (30).

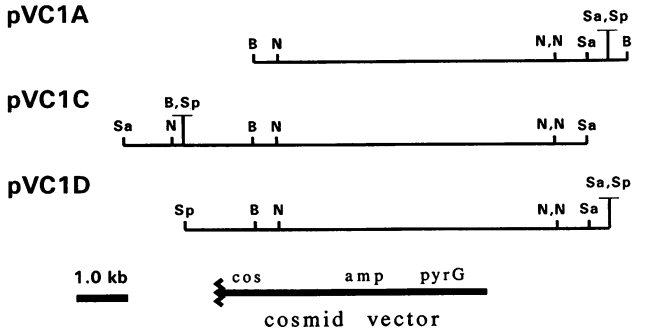

FIG. 3. DNA fragments obtained by marker rescue from an *A. parasiticus* CS10 aflatoxin-producing transformant. The region corresponding to the cosmid vector pBZ5 is represented at the bottom of the figure. Plasmid pVC1B is not shown. B, *Bgl*II; N, *Nde*I; Sa, *Sal*I; Sp, *Sph*I; Cos, cohesive site in lambda.

Transformation of *A. parasiticus* CS10 with pVC1A-D. Plasmids pVC1A, pVC1B, pVC1C, and pVC1D were used to transform *A. parasiticus* CS10 to test for the presence of the *pyrG* and *ver-1* genes. Pyrimidine-prototrophic transformants were obtained with all plasmids except pVC1B. The percentages of Afl$^+$ isolates in the total number of *pyrG*$^+$ transformants were 29, 20, and 18% for pVC1A, pVC1C, and pVC1D, respectively. No Afl$^+$ isolates have ever been detected in transformations with pPG3J and pBZ5, which contain only the *pyrG* gene and vector DNA. Complementation of the *ver-1* gene with the rescued fragments eliminated the necessity to conduct in situ colony hybridization of the cosmid genomic library with the rescued fragments as probes.

Analysis of *A. parasiticus* Afl$^+$ transformants. Five clones transformed with pVC1A (designated P1 through P5) that demonstrated blue fluorescence on CAM and five transformants also transformed with pVC1A (designated N1 through N5) that lacked blue fluorescence were subcultured onto CAM for further analysis. Upon visual inspection of the cultures, the Afl$^+$ transformants (P1 through P5) were easily distinguishable from the Afl$^-$ strains by day 2 of incubation. All Afl$^+$ transformants except P2 accumulated little detectable yellow pigmentation in the mycelium, whereas the Afl$^-$

TABLE 1. Sclerotium production of various *A. parasiticus* strains grown on PDA medium for 10 days

Strain	Growth temp (°C)	No. of sclerotia/ 10 cm^2	Avg diameter (μm)[a]
P1	30	130	317 ± 53
	37	—[b]	
P2	30	0	
	37	—	
P3	30	180	360 ± 56
	37	—	
P4	30	7	332 ± 51
	37	—	
P5	30	120	329 ± 50
	37	—	
N1 through N5	30	0	
	37	—	
NRRL 5862	30	69	338 ± 55
	37	32	341 ± 55
ATCC 36537	30	0	
	37	33	372 ± 54

[a] Between 32 and 100 sclerotia were chosen at random for each strain.
[b] —, growth was negligible (colony diameter, <0.5 cm) at 37°C for all *pyrG*$^+$ transformants tested.

isolates (N1 through N5) accumulated abundant quantities of yellow pigment. This yellow pigment has been confirmed by both TLC and ELISA to contain predominantly VA (52). Under longwave UV light, all of the Afl$^+$ transformants produced a blue fluorescent pigment by day 3 of incubation at 30°C; no blue fluorescent pigment was detectable in clones N1 through N5. One Afl$^+$ transformant, P2, produced a small quantity of a blue fluorescent compound and accumulated less yellow pigment than did the Afl$^-$ isolates.

By using TLC and direct competitive ELISA analysis (data not shown), it was confirmed that *A. parasiticus* P1, P3, P4, and P5 produced aflatoxin B_1 in amounts (approximately 20 [± 10] μg of aflatoxin per ml of filtrate) comparable to those in *A. parasiticus* NRRL 5862 grown under identical conditions. *A. parasiticus* P2 produced aflatoxin B_1 at an approximately 10-fold-lower level. No aflatoxins were detected (1 ng/ml is the minimum detectable level for the monoclonal antibody employed) in the filtrate from *A. parasiticus* N1, N2, N3, N4, or N5 or *A. parasiticus* ATCC 36537. TLC analysis demonstrated that VA was present in small amounts in the filtrates from all of the non-aflatoxin-producing strains, *A. parasiticus* ATCC 36537, and *A. parasiticus* P2. VA is not normally found in the filtrate, since it does not traverse the cell wall efficiently (42). However, it is likely that some of this pigment is released into the broth medium when the integrity of the cell wall is lost during cell death or as cells are physically disrupted by the glass beads.

Each of the Afl$^+$ and Afl$^-$ transformants was reinoculated onto PDA and grown for 10 days at 30 and 37°C. Sclerotia, which are composed of a highly pigmented dense mass of hyphae, were abundant at 30°C in the wild-type strain *A. parasiticus* NRRL 5862 and in all of the Afl$^+$ transformants except *A. parasiticus* P2, which did not produce any of these dormant structures (Table 1). No sclerotia were observed in any of the Afl$^-$ transformants or in the mutant strain *A. parasiticus* ATCC 36537 grown on PDA under identical conditions. However, *A. parasiticus* ATCC 36537 did produce sclerotia at 37°C, which is inhibitory to aflatoxin biosynthesis (and VA biosynthesis) (47); no yellow pigmentation of the mycelium was evident at this temperature. Transformants could not be analyzed at 37°C because they did not grow, presumably because the *pyrG* gene used for transformation was not effectively expressed at this temperature.

Southern analysis was performed on *Bam*HI-digested genomic DNA from *A. parasiticus* P1 through P5 and CS10 with the 2.6-kb *Pvu*I-*Sph*I *ver-1*-containing restriction endonuclease fragment as a probe (data not shown). One additional hybridization band that did not appear in the *A. parasiticus* CS10 control was observed in approximately the same location for *A. parasiticus* P1 through P5. The relative intensities of these bands suggested that only one or at most two additional copies of transforming *ver-1* DNA had integrated into the genome. Additional studies will be performed to determine whether integration of *ver-1* at a specific locus is required for full complementation activity. Differences in the percentage of Afl$^+$ isolates among the total number of transformants may be a function of the various *ver-1*-containing fragments with preferred sites of integration that may or may not complement the *ver-1* mutation. Since the procedure for selection of transformants was based on a functional *pyrG* gene, it is probable that integration of the transforming plasmid into the chromosome resulted in nonfunctional copies of the *ver-1* gene and therefore less than 100% restoration of aflatoxin biosynthesis among all of the transformants.

Localization of mRNA transcripts encoded by the *ver-1* gene fragment. We were able to predict the approximate location of the region responsible for the *ver-1* complementation activity by identifying the smallest common restriction endonuclease fragment present in the *ver-1*-complementing plasmids pVC1A, pVC1C, and pVC1D. Since we knew from control transformations that Afl$^+$-complementing activity was not contained on the cosmid vector pBZ5, we could determine that it must lie between the *pyrG* gene in the vector and the first *Sal*I restriction endonuclease site within the insert (Fig. 3).

Northern analysis was used to determine the sizes and locations of transcription units contained on the Afl$^+$-complementing DNA fragments (Fig. 4). Overlapping restriction fragments were used as DNA probes for hybridization to RNA purified from *A. parasiticus* NRRL 5862 or *A. parasiticus* ATTC 36537. No significant differences in hybridization patterns between the two strains were noted. Three different RNAs (0.6, 1.0, and 1.5 kb) were detected by Northern analyses with radiolabelled probes made from the 3.4-kb *Cla*I-*Bgl*II restriction fragment. On the basis of hybridization patterns observed with the DNA probes, it was predicted that the 0.6-kb RNA was encoded primarily on the 0.5-kb *Hin*dIII restriction fragment with a small portion of the transcription coding region extending into the 0.4-kb *Ava*I-*Hin*dIII fragment. The region encoding the 1.0-kb RNA was localized to the 1.8-kb *Eco*RI-*Sal*I restriction fragment. Finally, it was estimated that only part of the 1.5-kb RNA transcript was encoded on the 0.3-kb *Cla*I-*Pvu*I restriction fragment.

Location of the *ver-1* transcript. It was initially postulated that either the 1.0-kb transcript or the 0.6-kb transcript was responsible for complementation of the *ver-1* mutation in *A. parasiticus* CS10. We did not believe that the region encoding the 1.5-kb transcript would be able to complement such

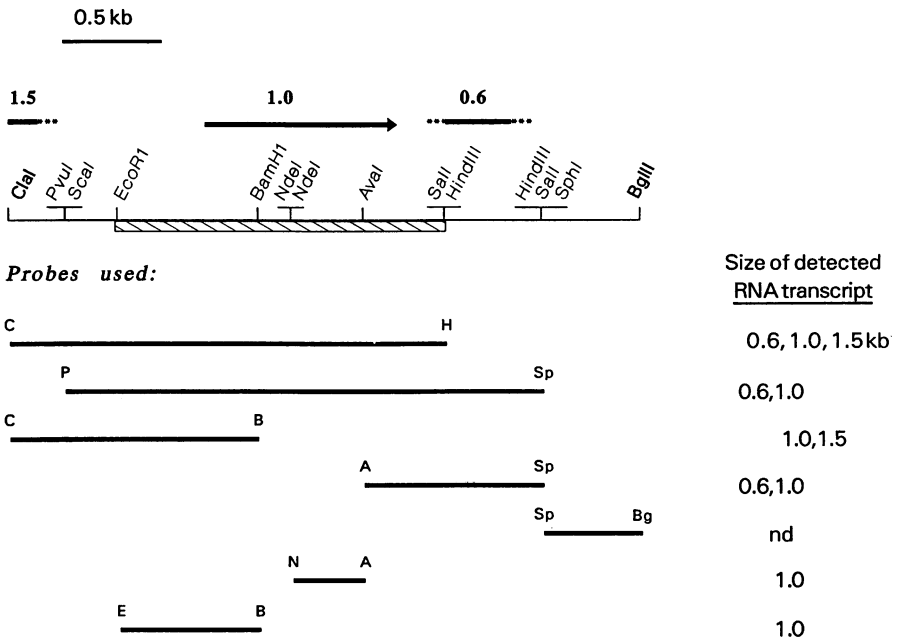

FIG. 4. Summary of Northern analyses with probes made from DNA fragments subcloned from the 3.4-kb *Cla*I-*Bgl*II region of pVC1A. Polyadenylated RNA was used for hybridization to the labelled fragments. The approximate locations of transcripts are represented by solid bars at the top of the diagram. The arrows designate the directions of transcription. Asterisks on the ends of the bars reflect approximate locations of transcript ends. The shaded area beneath the 1.8-kb *Eco*RI-*Hin*dIII shows the smallest fragment to functionally complement *ver-1*. nd, none detected.

a high percentage of transformants to Afl$^+$ when only a small part of the RNA coding region (less than 0.3 kb) was present. In addition, the region encoding the entire 0.6-kb transcript (1.9-kb *Bam*HI-*Bgl*II fragment cloned into pBZ5) was unable to restore *A. parasiticus* CS10 to Afl$^+$. By a process of elimination, it was predicted that the 1.0-kb transcript was responsible for complementing the *ver-1* mutation. This hypothesis was confirmed when the 1.8-kb *Eco*RI-*Sal*I fragment from pVC1A cloned into pBluescriptII KS− (pBSV4) restored aflatoxin production to 5 (11%) of a total of 46 *pyrG*$^+$ *A. parasiticus* CS10 transformants after cotransformation with the *pyrG*-containing plasmid pBP28. Although pBSV4 does contain DNA encoding a part of the 0.6-kb transcript, a DNA fragment encoding the entire 0.6-kb RNA was unable to complement the *ver-1* mutation.

Analysis of transcripts from the *ver-1* gene. Strand-specific riboprobes were used in Northern analysis of *A. parasiticus* NRRL 5862 total RNA. The direction of transcription of the *ver-1* gene was determined to proceed from the *Bam*HI restriction endonuclease site to the *Ava*I site (data not shown). The approximate locations of the transcription initiation site and polyadenylation site on the *ver-1* transcript were determined by an RNase protection assay. Several protected fragments of similar sizes were present in both mRNA and tRNA control lanes (Fig. 5). However, a unique 290-bp fragment was protected in the mRNA 5′-end assay and not the tRNA control, suggesting that transcription of the *ver-1* RNA initiates approximately 290 bp upstream from the *Bam*HI restriction endonuclease site in the *ver-1* gene. Similarly, the 3′ end of the *ver-1* transcript (polyadenylation site) was localized to a region approximately 230 bp downstream from the *Ava*I restriction endonuclease site. These data correlate well with a total transcript size of 1.0 kb observed in Northern analysis and the location of ends of the transcript predicted by cDNA sequence analysis.

Nucleotide sequence analysis. The nucleotide sequence of genomic DNA and cDNA from the entire *ver-1*-coding region was determined. A short sequence characteristic of a Hogness box (TATATAT) was observed in the same general region that a transcription initiation site was localized by

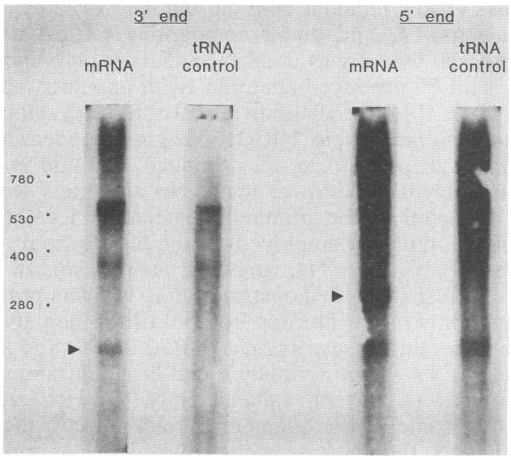

FIG. 5. Assay of RNase protection of 5′ and 3′ ends of the *ver-1* transcript. Radiolabelled riboprobes were transcribed from the 0.4-kb *Ava*I-*Hin*dIII and 0.7-kb *Eco*RI-*Bam*HI restriction fragments for 3′ and 5′ end analysis, respectively, and hybridized to polyadenylated RNA or tRNA (control) isolated from *A. parasiticus*. Samples were treated with RNase, and protected fragments were separated by polyacrylamide gel electrophoresis under denaturing conditions. Unique 230- and 290-bp fragments (arrows) were protected in 3′ and 5′ reactions, respectively. The locations and sizes (nucleotides) of RNA reference standards are included to the left.

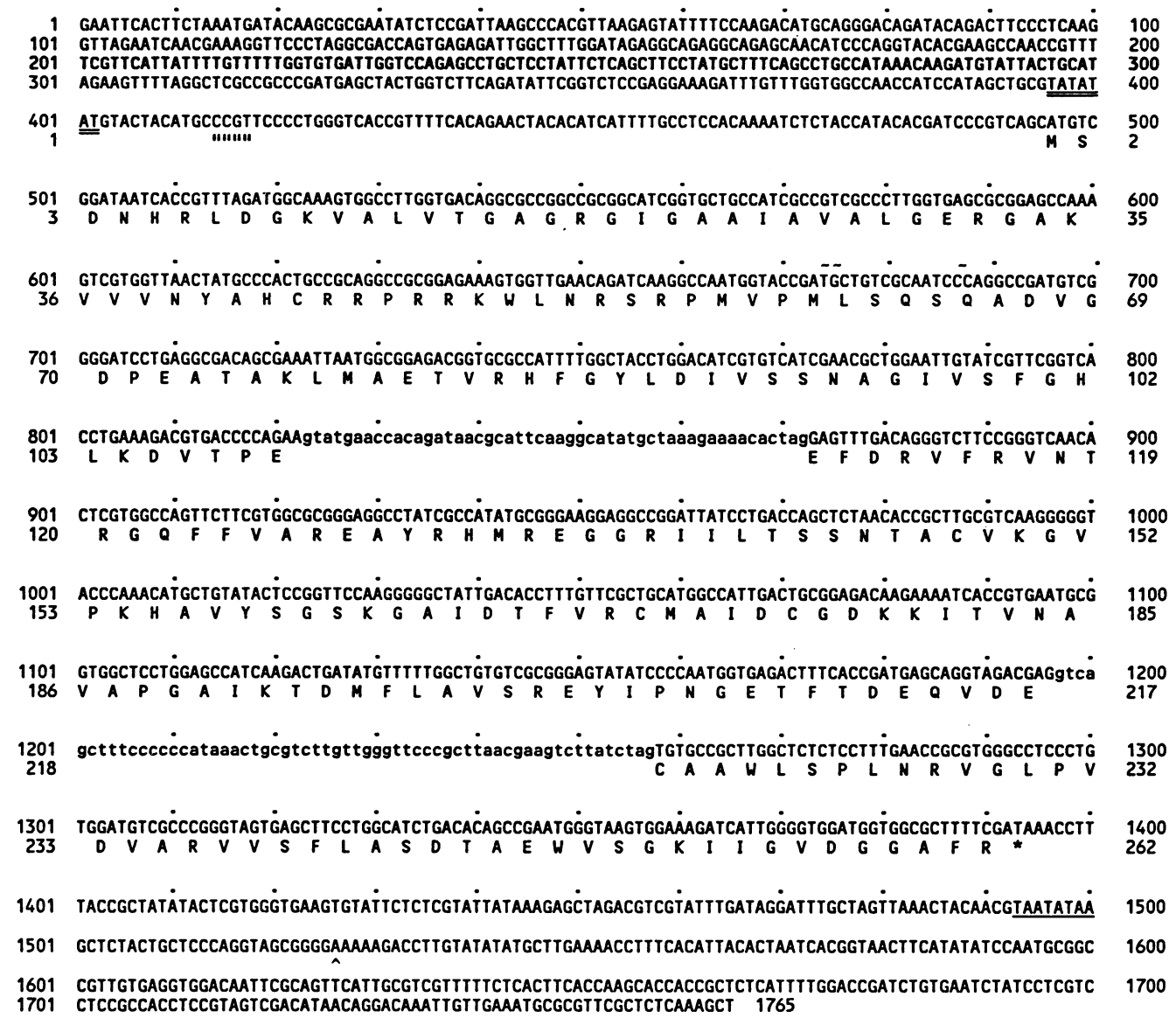

FIG. 6. Nucleotide sequence of the 1.8-kb *Eco*RI-*Hin*dIII restriction fragment containing the *ver-1* gene. The predicted amino acid sequence is shown below the corresponding codon. Probable consensus sequences are underlined as follows: ═══, Hogness box; ____, polyadenylation motif. " " ", region of transcription initiation (predicted by RNase protection); ^, polyadenylation site (predicted by RNase protection and confirmed by cDNA sequence); -, nucleotides detected only in the cDNA sequence.

RNase protection (Fig. 6). The two closest ATG initiation codons downstream from the TATA box were 8 and 90 nucleotides downstream. The latter, which was followed by a long open reading frame, was chosen as the most likely translation initiation codon. Protein coding regions were predicted by using the Wisconsin Genetics Computer Group software to test the randomness of codons and codon preference within the 1.8-kb *Eco*RI-*Hin*dIII restriction fragment. The locations of intervening sequences could then be predicted by using this information with open reading frame analyses. Splice junctions for introns were tentatively identified by comparing consensus sequences of intron boundaries and internal regions associated with lariat formation in *Aspergillus* spp. (31). Two introns were predicted for the *A. parasiticus ver-1* sequence; these introns would allow translation consistent with the predicted open reading frames. The locations and lengths (50 and 61 nucleotides) of the introns were confirmed by analysis of a cDNA clone. The codon TAA in the final open reading frame (132 nucleotides in length) was believed to be the translation termination codon because it was located approximately 130 nucleotides upstream from the location of the polyadenylation site that was predicted by RNase protection and confirmed by cDNA sequence analysis. No other reasonable open reading frames could be found beyond this point. The putative spliced *ver-1* sequence encodes a predicted polypeptide sequence of 262 amino acids.

In a search of the EMBL and GenBank data base libraries with the Wisconsin Genetics Computer Group FastA protocol, the predicted amino acid sequence of the *ver-1* protein

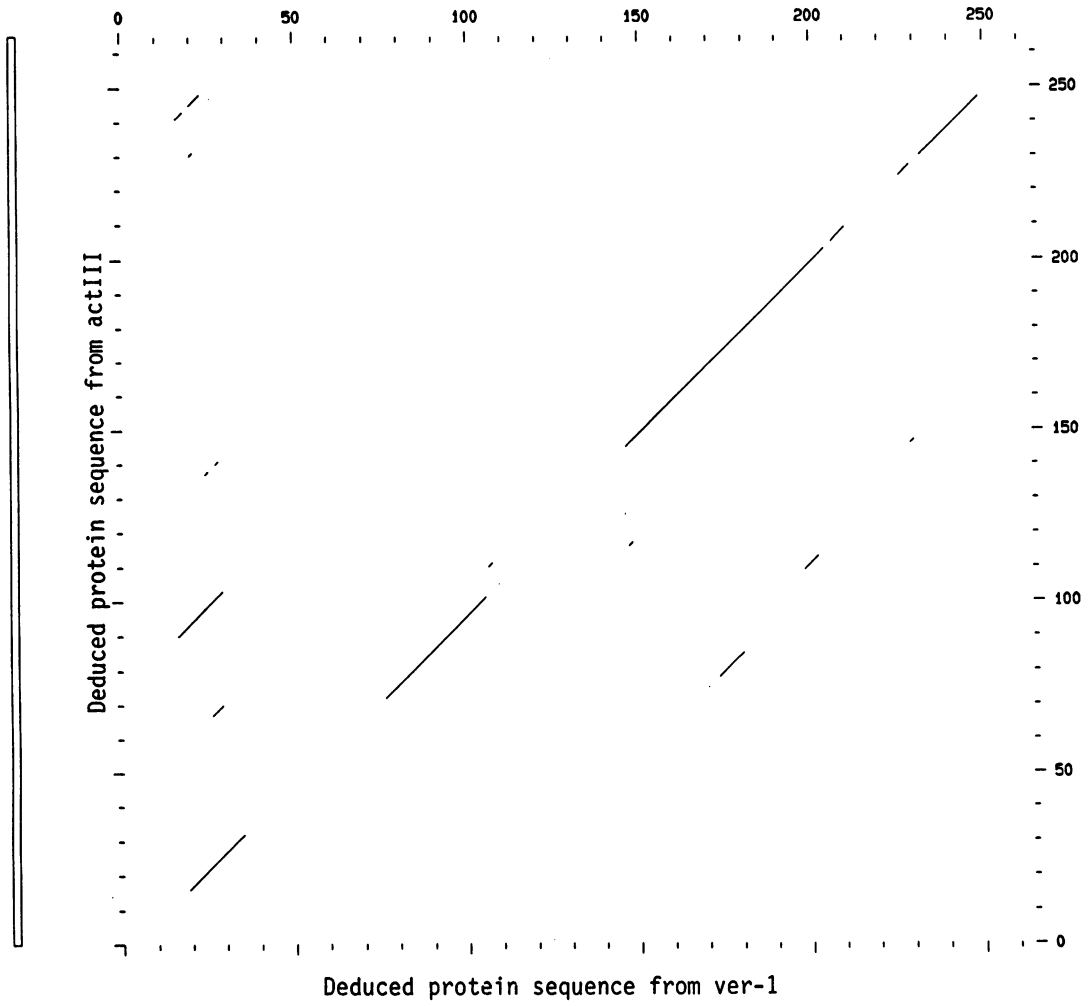

FIG. 7. Dot plot analysis comparing the deduced peptide sequence from the *A. parasiticus ver-1* gene and the deduced peptide sequence from the *Streptomyces actIII* gene (ketoreductase). Window, 30; stringency, 15.

showed abundant identity with numerous reductases and dehydrogenases. A dot plot comparison (Fig. 7) of the predicted *ver-1* amino acid sequence with the known polypeptide sequence from the *Streptomyces coelicolor actIII* gene (32), which encodes a ketoreductase associated with biosynthesis of the polyketide actinorhodin, demonstrates the extent of identity between these proteins. Similar dot plots were observed when peptide sequences from the *Azospirillum brasilense nodG* gene (26), which encodes a ketoreductase associated with plant nodulation, and the *Bacillus megaterium gdhA* gene (34), which encodes a glucose dehydrogenase, were compared with the predicted *ver-1* amino acid sequence. All three proteins used in these comparisons were nearly identical in size (within 1 or 2 amino acids) to the predicted *ver-1* protein. Best-fit analysis with the amino acid sequence from *A. parasiticus ver-1* determined an average 52% similarity (includes conservative substitutions) and 33% identity with all three of the proteins over their entire length. A predicted adenine nucleotide binding motif, Gly-X-Gly-X-X-Ala-12X-Lys consistent with NAD(P) or ATP binding proteins (35, 40) can be found starting at Gly-18.

DISCUSSION

The data strongly suggest that the *ver-1* gene is associated with the conversion of VA to ST because it complements Afl^- mutants, which are unable to carry out this biotransformation step. Since this complex conversion of an anthraquinone moiety to a xanthone structure is believed to occur through a number of enzymatic steps, it is probable that the *ver-1* polypeptide must cooperate with the products of other genes. Nucleotide sequence analysis proved to be extremely useful in predicting that the *ver-1* gene encodes a protein that has enzymatic activity associated with this bioconversion. The predicted *ver-1* protein displays significant conservation of size and amino acid sequence with reductases and dehydrogenases involved in modification of ring structures. Further support of this predicted enzymatic function is the hypothesis of an NADPH-dependent reductase involved in the conversion of VA to ST (9, 27). After complementation with the *ver-1* gene in Afl^- mutants, the biosynthesis of aflatoxins B_2 and G_2 in addition to aflatoxins B_1 and G_1 was also restored (7a). This suggests that the product of the *ver-1* gene is also associated with the conver-

sion of versicolorin B to dihydrosterigmatocystin (43, 58). This proposal is consistent with theories that speculate that one set of enzymes carries out parallel functions on alternative aflatoxin intermediates, in a branched pathway which allows for the synthesis of aflatoxins B_1 and G_1 in one branch and aflatoxins B_2 and G_2 in the other branch (9).

Although we cannot conclusively rule out the possibility that *ver-1* encodes a suppressor activity, the data strongly suggest that the restoration of aflatoxin biosynthesis in *ver-1* transformants is due to a direct complementation of a missing enzyme activity. (i) Nucleotide sequence analysis predicts a reductase function for the product of the *ver-1* gene. (ii) No Afl^+ transformant has ever been observed when *A. parasiticus* CS10 is transformed with only the *pyrG* gene, suggesting that the *ver-1* mutation is quite stable (i.e., it is improbable that we are seeing reversion of a mutation). (iii) VA is converted to aflatoxin in Afl^+ transformants at an efficiency comparable to that of the wild-type strain. This efficiency would not be anticipated in transformants with only one additional copy of a gene encoding an enzyme that fortuitously converts VA to ST. (iv) RNA transcripts from the *ver-1* gene accumulate only during idiophase (54a), suggesting that the gene functions in secondary metabolism.

We observed that $pyrG^+$ Afl^+ transformants of *A. parasiticus* CS10 produce abundant levels of sclerotia on PDA, whereas $pyrG^+$ Afl^+ transformants do not produce sclerotia under identical growth conditions. This intriguing observation strongly supports previous hypotheses (6, 20, 21) that sclerotium development and aflatoxin biosynthesis in *A. parasiticus* are associated. However, it is not clear whether regulation of these two processes are somehow interrelated or whether there is also a direct functional relationship (i.e., aflatoxins or pathway intermediates affect sclerotia development). The appearance of sclerotia in *A. parasiticus* ATCC 36537 grown at 37°C (a temperature that inhibits aflatoxin biosynthesis) suggests that the restored sclerotium production is a result of the disappearance of VA. If VA is inhibitory to sclerotium production, then preventing VA accumulation either by increasing its conversion to aflatoxin B_1 or by suppressing its synthesis should restore sclerotia. Secondary metabolism in *Streptomyces* spp. is also believed to be linked with morphological development (36). This hypothesis was supported by the isolation of a pleiotropic gene (*afsB*) that positively regulates the biosynthesis of A factor, the polyketide-derived antibiotic actinorhodin, and undecylprodigiosin. However, our data suggest that *ver-1* is a structural gene encoding a ketoreductase. It seems improbable that a gene product with enzymatic activity would also have direct regulatory functions. However, aflatoxin B_1 or pathway intermediates that are produced in $pyrG^+$ Afl^+ *A. parasiticus* CS10 transformants could have a direct or indirect effect on regulatory proteins involved in aflatoxin biosynthesis and/or sclerotium development.

Parasexual analysis with Afl^- mutants in *A. parasiticus* has generated conflicting results regarding the genetic linkage of *nor-1* and *ver-1* (7, 11). We have identified a cosmid that hybridizes to both the *nor-1* and *ver-1* genes (data not shown). It is possible that random association of separate restriction fragments carrying *nor-1* and *ver-1* occurred during construction of the cosmid library. However, the low probability of such an event supports a physical linkage of the *ver-1* and *nor-1* genes in the *A. parasiticus* genome. Although structural genes are infrequently linked in aspergilli, there are numerous exceptions to this pattern (31). The apparent close proximity of these two genes suggests that many of the aflatoxin genes are tightly clustered, as are the polyketide-derived pigments in *Streptomyces* spp. (12, 25, 32, 54). In support of this hypothesis, Papa (49) concluded that 9 of 11 aflatoxin variants of *A. flavus* were in linkage group VII, which contains the *nor-1* locus. The remaining two were localized to linkage groups II and VIII.

Genetic complementation of Afl^- mutants of *A. parasiticus* was shown to be an effective strategy for the isolation of a gene associated with aflatoxin biosynthesis. A similar approach has been used successfully to clone other aflatoxin-associated genes in *A. parasiticus* (13) and *A. flavus* (50). Recently, it was reported that differential screening of an *A. parasiticus* genomic DNA library was used to isolate genes whose expression is correlated with production of the secondary metabolite aflatoxin B_1 (29). It will be interesting to learn whether these genes are indeed associated with aflatoxin production, since conditions favoring this biosynthesis may also lead to production of other secondary metabolites and major changes in the expression of RNA species (57) and proteins (48) observed during development in aspergilli.

Future research is being directed toward analyses of the regulation of gene expression of aflatoxin biosynthetic genes. We are particularly interested in the relationship between aflatoxin biosynthesis and developmental morphogenesis. Disruptions of aflatoxin-associated genes are currently being used to study this relationship and also to develop Afl^- strains that can be used in biocompetition in the field to reduce or eliminate aflatoxin contamination of crops. We are also examining the genes that are linked to the *nor-1* and *ver-1* genes to determine whether they are also associated with aflatoxin biosynthesis.

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ADDENDUM IN PROOF

As a result of recent analysis of the linkage between *nor-1* and *ver-1* in *A. parasiticus*, we have identified a new DNA fragment which hybridizes to a *ver-1* internal probe. The extent of similarity of the DNA fragment to *ver-1* is being investigated.

REFERENCES

1. **Adye, J., and R. I. Mateleles.** 1964. Incorporation of labelled compounds into aflatoxin. Biochim. Biophys. Acta **86**:418–420.
2. **Anderson, J. A., and C. H. Chung.** 1990. Conversion of versiconal acetate to versiconal and versicolorin C in extracts from *Aspergillus parasiticus*. Mycopathologia **110**:31–35.
3. **Anderson, J. A., C. H. Chung, and S. U. Cho.** 1990. Versicolorin A hemiacetal, hydroxydihydrosterigmatocystin, and aflatoxin G_{2a} reductase activity in extracts from *Aspergillus parasiticus*. Mycopathologia **111**:39–45.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
5. **Bennett, J. W., and L. A. Goldblatt.** 1973. The isolation of mutants of *Aspergillus flavus* and *A. parasiticus* with altered aflatoxin producing ability. Sabouraudia **11**:235–241.
6. **Bennett, J. W., P. M. Leong, S. Kruger, and D. Keyes.** 1986. Sclerotial and low aflatoxigenic morphological variants from haploid and diploid *Aspergillus parasiticus*. Experientia **42**:848–851.
7. **Bennett, J. W., C. H. Vinnett, and W. R. Goynes, Jr.** 1980.

Aspects of parasexual analysis in *Aspergillus parasiticus*. Can. J. Microbiol. **26:**706–713.

7a. **Bhatnagar, D.** Personal communication.

8. **Bhatnagar, D., and T. E. Cleveland.** 1990. Purification and characterization of a reductase from *Aspergillus parasiticus* SRRC 2043 involved in aflatoxin biosynthesis. FASEB J. **4:**A2164.

9. **Bhatnagar, D., K. C. Erlich, and T. E. Cleveland.** 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites and their control by environmental factors, p. 255–286. *In* D. Bhatnagar, E. B. Lillehoj, and D. K. Arora (ed.), Handbook of applied mycology, vol 5. Marcel Dekker, Inc., New York.

10. **Bhatnagar, D., A. H. J. Ullah, and T. E. Cleveland.** 1988. Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. Prep. Biochem. **18:**321–349.

11. **Bradshaw, R. E., J. W. Bennett, and J. F. Peberdy.** 1983. Parasexual analysis of *Aspergillus parasiticus*. J. Gen. Microbiol. **129:**2117–2123.

12. **Caballero, J. L., E. Martinez, F. Malpartida, and D. A. Hopwood.** 1991. Organisation and function of the *act*VA region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. **230:**401–412.

13. **Chang, P. K., C. S. Skory, and J. E. Linz.** 1992. Cloning of a gene associated with aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*. Curr. Gen. **21:**231–233.

14. **Chu, F. S.** 1991. Mycotoxins: food contamination, mechanisms, carcinogenic potential, and preventative measures. Mutat. Res. **259:**291–306.

15. **Chu, F. S., M. S. Hsia, and P. S. Sun.** 1977. Preparation and characterization of aflatoxin B_1-1-(O-carboxymethyl) oxime. J. Assoc. Off. Anal. Chem. **60:**791–794.

16. **Chuturgoon, A. A., M. F. Dutton, and R. K. Berry.** 1990. The preparation of an enzyme associated with aflatoxin biosynthesis by affinity chromatography. Biochem. Biophys. Res. Commun. **166:**38–42.

17. **Cleveland, T. E.** 1987. Appearance of enzyme activities catalyzing conversion of sterigmatocystin to aflatoxin B_1, in late-growth phase *Aspergillus parasiticus* cultures. Appl. Environ. Microbiol. **53:**1711–1713.

18. **Cleveland, T. E.** 1989. Conversion of dihydro-O-methyl-sterigmatocystin to aflatoxin B_2 by *Aspergillus parasiticus*. Arch. Environ. Contam. Toxicol. **18:**429–433.

19. **Cole, R. J., J. W. Dorner, P. D. Blankenship, and B. Horn.** 1991. The use of biocompetitive agents for preharvest aflatoxin control in peanuts. Abstr. USDA-ARS aflatoxin elimination workshop, Atlanta, Ga.

20. **Cotty, P. J.** 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: influence of pH. Phytopathology **78:**1250–1253.

21. **Cotty, P. J.** 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology **79:**808–814.

22. **Cotty, P. J.** 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. **74:**233–235.

23. **Cotty, P. J., and D. J. Daigle.** 1991. Update on *Aspergillus flavus* populations and prevention of aflatoxin through intraspecific competition. Abstr. USDA-ARS aflatoxin elimination workshop, Atlanta, Ga.

24. **Davis, N. D., S. K. Iyer, and U. L. Diener.** 1987. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. **53:**1593–1595.

25. **Davis, N. K., and K. F. Chater.** 1990. Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. Mol. Microbiol. **4:**1679–1691.

26. **Delledonne, M., R. Porcari, and C. Fogher.** 1990. Nucleotide sequence of the *nod*G gene of *Azospirillum brasilense*. Nucleic Acids Res. **18:**6435.

27. **Dutton, M. F.** 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. **53:**274–295.

28. **Ehrlich, K.** 1987. Effect on aflatoxin production of competition between wild-type and mutant strains of *Aspergillus parasiticus*. Mycopathologia **97:**93–96.

29. **Feng, G. H., F. S. Chu, and T. J. Leonard.** 1992. Molecular cloning of genes related to aflatoxin biosynthesis by differential screening. Appl. Environ. Microbiol. **58:**455–460.

30. **Gibson, T. J., A. R. Coulson, J. E. Sulston, and P. R. Little.** 1987. Lorist 2, a cosmid with transcriptional terminators insulating vector genes from interference by promoter within the insert: effect on DNA yield and cloned insert frequency. Gene **53:**275–281.

31. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1987. The structure and organization of nuclear genes of filamentous fungi, p. 95–138. *In* J. R. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.

32. **Hallam, S., F. Malpartida, and D. Hopwood.** 1988. Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene **74:**305–320.

33. **Heathcote, J. G., and J. R. Hibbert.** 1978. Aflatoxins: chemical and biological aspects. Elsevier Scientific, New York.

34. **Heilmann, J. J., H. J. Maegert, and H. G. Gassen.** 1988. Identification and isolation of glucose dehydrogenase genes of *Bacillus megaterium* M1286 and their expression in *Escherichia coli*. Eur. J. Biochem. **174:**485–490.

35. **Hopwood, D. A., and D. H. Sherman.** 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu. Rev. Gen. **24:**37–66.

36. **Horinouchi, S., H. Suzuki, and T. Beppu.** 1986. Nucleotide sequence of *afsB*, a pleiotropic gene involved in secondary metabolism in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bacteriol. **168:**257–269.

37. **Horng, J. S., P. K. Chang, J. J. Pestka, and J. E. Linz.** 1990. Development of a homologous transformation system for *Aspergillus parasiticus* with the gene encoding nitrate reductase. Appl. Environ. Microbiol. **55:**2561–2568.

38. **Hsieh, D. P. H., C. C. Wan, and J. A. Billington.** 1989. A versiconal hemiacetal acetate converting enzyme in aflatoxin biosynthesis. Mycopathologia **107:**121–126.

39. **Jelinek, C. F., A. E. Pohland, and G. E. Wood.** 1989. Worldwide occurrence of mycotoxins in foods and feeds—an update. J. Assoc. Off. Anal. Chem. **72:**223–230.

40. **Kamps, M. P., S. S. Taylor, and B. M. Sefton.** 1984. Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. Nature (London) **310:**589–591.

41. **Keller, N., H. C. Dischinger, Jr., D. Bhatnagar, T. E. Cleveland, and A. H. J. Ullah.** 1991. Purification of a second methyltransferase active in the aflatoxin biosynthetic pathway. FASEB J. **5:**2623.

42. **Lee, L. S., J. W. Bennett, A. F. Cucullu, and J. B. Stanley.** 1975. Synthesis of versicolorin A by a mutant strain of *Aspergillus parasiticus* deficient in aflatoxin production. J. Agr. Food Chem. **23:**1132–1134.

43. **Lin, B. K., and J. A. Anderson.** 1992. Purification and properties of versiconal cyclase from *Aspergillus parasiticus*. Arch. Biochem. Biophys. **293:**67–70.

44. **Lin, Y. C., J. C. Ayres, and P. E. Koehler.** 1980. Influence of temperature cycling on the production of aflatoxins B1 and G1 by *Aspergillus parasiticus*. Appl. Environ. Microbiol. **40:**333–336.

45. **Lloyd, A. T., and P. M. Sharp.** 1991. Codon usage in *Aspergillus nidulans*. Mol. Gen. Genet. **230:**288–294.

46. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

47. **Niehaus, W. G., Jr.** 1989. Versicolorin synthesis by *Aspergillus parasiticus*: regulation by temperature and zinc. Exp. Mycol. **13:**20–26.

48. **Novak, L. A., and L. M. Kohn.** 1990. Developmental proteins in *Aspergillus*. Exp. Mycol. **14:**339–350.

49. **Papa, K. E.** 1984. Genetics of *Aspergillus flavus*: linkage of aflatoxin mutants. Can. J. Microbiol. **30:**68–73.

50. **Payne, G. A.** 1991. Characterization of genes for aflatoxin biosynthesis. Abstr. USDA-ARS aflatoxin elimination work-

shop, Atlanta, Ga.
51. **Pestka, J. J.** 1988. Enhanced surveillance of foodborne mycotoxins by immunochemical assay. J. Assoc. Off. Anal. Chem. **71**:1075–1081.
52. **Reynolds, G. N., and J. J. Pestka.** 1991. Enzyme-linked immunosorbent assay of versicolorin A and related aflatoxin biosynthetic precursors. J. Food Prot. **54**:105–108.
53. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. **74**:5463–5467.
54. **Sherman, D. H., F. Malpartida, M. J. Bibb, H. M. Kieser, M. J. Bibb, and D. A. Hopwood.** 1989. Structure and deduced function of the granaticin-producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tu22. EMBO J. **8**:2717–2725.
54a. **Skory, C. D.** Unpublished data.
55. **Skory, C. D., P. K. Chang, and J. Linz.** 1991. Isolation of a gene involved in the aflatoxin biosynthetic pathway of *Aspergillus parasiticus*. Abstr. 91st Annu. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
56. **Skory, C. D., J. S. Horng, J. J. Pestka, and J. E. Linz.** 1990. Transformation of *Aspergillus parasiticus* with the homologous gene (*pyrG*) involved in pyrimidine biosynthesis. Appl. Environ. Microbiol. **56**:3315–3320.
57. **Timberlake, W. E.** 1990. Molecular genetics of *Aspergillus* development. Annu. Rev. Genet. **24**:5–36.
58. **Yabe, K., Y. Ando, and T. Hamasaki.** 1991. Desaturase activity in the branching step between aflatoxins B_1 and G_1 and aflatoxins B_2 and G_2. Agric. Biol. Chem. **55**:1907–1911.
59. **Yabe, K., Y. Ando, J. Hashimoto, and T. Hamasaki.** 1989. Two distinct *O*-methyltransferases in aflatoxin biosynthesis. Appl. Environ. Microbiol. **55**:2172–2177.
60. **Yabe, K., Y. Nakamura, H. Nakajima, Y. Ando, and T. Hamasaki.** 1991. Enzymatic conversion of norsolorinic acid to averufin in aflatoxin biosynthesis. Appl. Environ. Microbiol. **57**:1340–1345.