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Comparative Mapping of Aflatoxin Pathway Gene Clusters in *Aspergillus parasiticus* and *Aspergillus flavus*

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Aflatoxins are toxic and carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are synthesized by condensation of acetate units; their synthesis is estimated to involve at least 16 different enzymes. In this study we have shown that at least nine genes involved in the aflatoxin biosynthetic pathway are located within a 60-kb DNA fragment. Four of these genes, *nor-1*, *aflR*, *ver-1*, and *omtA* (previously named *omt-1*), have been cloned in *A. flavus* and *A. parasiticus*. In addition, five other genes, *pksA*, *uvm8*, *aad*, *ord-1*, and *ord-2* have been recently cloned in *A. parasiticus*. The *pksA*, *aad*, and *uvm8* genes exhibit sequence homologies to polyketide synthase, aryl-alcohol dehydrogenase, and fatty acid synthase genes, respectively. The cDNA sequences of *ord-1* and *ord-2* genes, which may be involved in later steps of aflatoxin biosynthesis, have been determined; the *ord-1* gene product exhibits homology to cytochrome P-450-type enzymes. By characterizing the overlapping regions of the DNA inserts in different cosmid and lambda DNA clones, we have determined the order of these aflatoxin pathway genes within this 60-kb DNA region to be *pksA*, *nor-1*, *uvm8*, *aflR*, *aad*, *ver-1*, *ord-1*, *ord-2*, and *omtA* in *A. parasiticus* and *nor-1*, *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA* in *A. flavus*. The order is related to the order in enzymatic steps required for aflatoxin biosynthesis. The physical distances (in kilobase pairs) and the directions of transcription of these genes have been determined for both aflatoxigenic species.

Aflatoxins B_1 and B_2 (Fig. 1) are secondary metabolites produced by the aflatoxigenic species of the filamentous fungi *Aspergillus flavus* Link and *A. parasiticus* Speare when they infect corn, cotton, peanuts, and tree nuts. These compounds are known to be toxic and carcinogenic to animals. A positive correlation between aflatoxin contamination of agricultural commodities and primary human hepatocellular carcinoma has been documented (14, 15, 20, 34, 35, 41). For this reason, efforts to reduce and eventually eliminate aflatoxin contamination from food and feed have been undertaken by scientists worldwide.

Extensive biochemical and genetic studies have been conducted to better understand the molecular regulation of aflatoxin biosynthesis (for reviews, see references 7, 24, and 36). The initial step in the biosynthetic pathway of aflatoxins is the condensation of acetate units to form norsolorinic acid (for reviews, see references 8 and 29). The generally accepted pathway is illustrated in Fig. 1 (9, 46, 58). It is estimated that at least 16 enzymes are involved in the bioconversion of norsolorinic acid to aflatoxins (for reviews, see references 8 and 29); some of the enzymes have been identified and purified (8, 10, 11, 21, 23, 33, 38, 42, 57, 59, 60), but only a few have been purified to homogeneity (4, 11, 38, 42). Some of the genes coding for enzymes involved in the aflatoxin biosynthesis have been cloned (16, 17, 19, 49, 52, 53, 62, 63). These include the *pksA* gene, which codes for a polyketide synthase (17, 55a), the *nor-1* gene, which codes for a reductase that converts norsolorinic acid to averantin (19), the *ver-1* gene, which is involved in the conversion of versicolorin A to sterigmatocystin (52), and the *omtA* gene (previously named *omt-1*), coding for an *S*-adenosylmethionine-dependent *O*-methyltransferase (5, 8, 11, 22, 25, 59) that converts sterigmatocystin to *O*-methylsterigmatocystin and dihydrosterigmatocystin to dihydro-*O*-methylsterigmatocystin. The *omtA* gene has been cloned and characterized for both *A. parasiticus* and *A. flavus* (62, 63). In addition to these structural genes, a regulatory gene, *aflR* (previously named *afl-2* for *A. flavus* and *apa-2* for *A. parasiticus*), that codes for a regulatory factor (AFLR protein) has been cloned (16, 49, 56) and was shown to be involved in the activation of pathway gene transcription (16, 18). The step for the conversion of *O*-methylsterigmatocystin and dihydro-*O*-methylsterigmatocystin to AFB_1 and AFB_2 appears to be catalyzed by an oxidoreductase enzyme complex, which may consist of two or more subunits (5, 6, 8, 22); genes that code for the polypeptides of this enzyme complex have not yet been characterized. In addition, a putative fatty acid synthase gene, *uvm8*, potentially involved in polyketide backbone synthesis, first identified by complementation of a UV mutation (44a) and a gene, *aad*, homologous to aryl-alcohol dehydrogenase potentially involved in an intermediate step of aflatoxin biosynthesis (14a) have been cloned.

Linkage of aflatoxin pathway genes was first evidenced in an *A. parasiticus* cosmid clone, NorA, that contains both *nor-1* and *ver-1* genes (52). A physical and transcriptional map of the 35-kb genomic DNA insert in cosmid NorA suggested that several genes involved particularly in the early stages of aflatoxin B_1 biosynthesis are clustered on one chromosome in *A. parasiticus* (55a). However, no complete physical maps involv-

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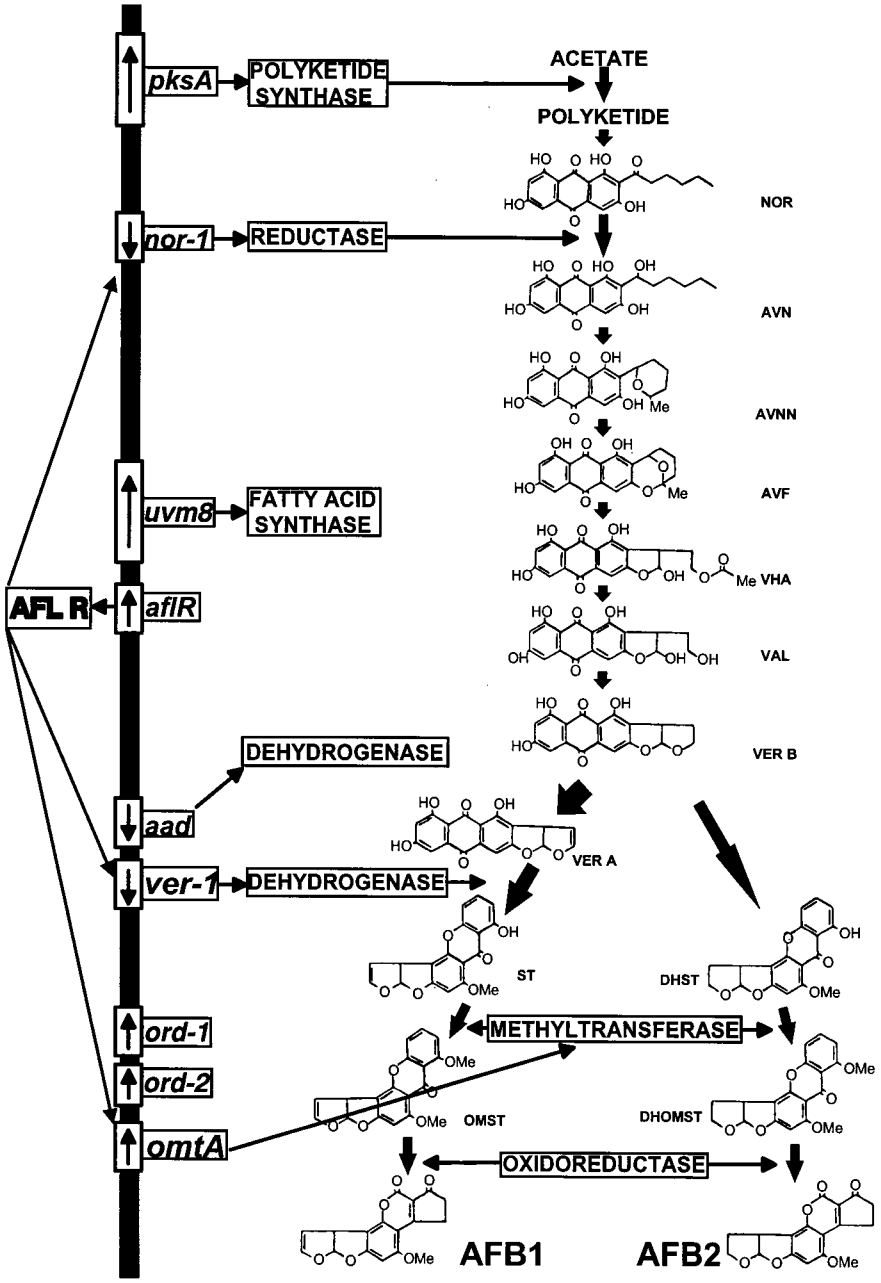

FIG. 1. Genes and enzymes in the aflatoxin B_1 and B_2 biosynthetic pathway. The generally accepted aflatoxin B_1 and B_2 biosynthetic pathway in *A. parasiticus* and *A. flavus*, enzymes for some specific conversion steps, and cloned genes are schematically presented. The regulatory gene, *aflR*, coding for the regulatory factor (AFLR protein), controls the expression of all of the characterized structural genes (*nor-1*, *ver-1*, and *omtA*). The *ver-1* gene product(s) has not been fully characterized; the catalytic steps in the aflatoxin biosynthetic pathway of the *uvm8* and the *aad* gene products, fatty acid synthase and a dehydrogenase, respectively, are not defined; and the *ord-1* and *ord-2* gene products are under investigation. The approximate sizes, relative locations, and directions of transcription of the identified genes, are indicated. Transcription of structural genes *nor-1*, *ver-1*, and *omtA* is regulated by AFLR. Abbreviations: NOR, norsolorinic acid; AVN, averantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VHOH versiconal; Ver B, versicolorin B; Ver A, versicolorin A; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB_1 aflatoxin B_1; AFB_2, aflatoxin B_2.

ing all of the identified aflatoxin pathway-related genes from both *A. parasiticus* and *A. flavus* have been reported. We present here the organization and arrangement of the aflatoxin pathway genes identified to date; these genes, responsible for both the early and later stages of aflatoxin biosynthesis, are clustered on a relatively small (60-kb) region of DNA. Based on the restriction enzyme analysis, physical maps have been generated for the two aflatoxigenic fungal species, *A. parasiticus* and *A. flavus*. The locations of the two additional transcripts that may encode enzymes involved in the later steps of the aflatoxin biosynthetic pathway are also presented.

MATERIALS AND METHODS

Fungal strains and culture conditions. Wild-type *A. parasiticus* SRRC 143 and *A. flavus* CRA01-2B (a β-tubulin mutant of NRRL 3357 that is resistant to

benomyl) were grown in darkness on potato dextrose agar (Difco Laboratories, Detroit, Mich.) plates for 7 days at 29°C. A 1.0-ml spore suspension (10^8 spores per ml) prepared from these cultures was transferred to a 2.8-liter Fernbach flask containing 1 liter of A & M growth medium (1) with sucrose substituted for glucose. Cultures were incubated on a rotary shaker (150 rpm) at 29°C. After 3 to 4 days, the medium was removed by vacuum filtration through Miracloth and the mycelia were frozen immediately in liquid nitrogen for later DNA isolation.

Bacterial and phage strains used. *Escherichia coli* P2392, LE392, and DH5α (Stratagene, La Jolla, Calif.) were used as host strains for lambda infection in lambda and cosmid genomic DNA library preparation. *E. coli* XL1-Blue MRF′ and helper phage (Stratagene) were used for the cDNA construction and excision of the cDNA insert from lambda vectors.

Chemicals and enzymes. Unless indicated otherwise, chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Luria broth base and NZY broth were purchased from GIBCO-BRL (Gaithersburg, Md.). Restriction endonucleases and corresponding buffers were purchased from Promega (Madison, Wis.) and were used according to the manufacturer's specifications.

Construction of genomic DNA libraries. The genomic DNA isolated from *A. parasiticus* SRRC 143 (NRRL 5862) was partially digested with *Sau*3AI, and 7- to 23-kb fragments were purified by gradient centrifugation. The purified DNA fragments were ligated to *Bam*HI arms of lambda EMBL3. An *A. parasiticus* cosmid DNA library constructed by ligation of 30- to 40-kb DNA fragments to the pBZ5 cosmid vector was also used for cosmid clone screening. The pBZ5 vector and library construction have been described elsewhere (52).

An *A. flavus* cosmid genomic DNA library was made with DNA isolated from aflatoxigenic *A. flavus* CRA01-2B. Genomic DNA partially digested with *Sau*3AI was ligated to the *Bam*HI site of the pAF1 cosmid vector. The ligated DNA constructs were packaged into lambda phage particles, and the cosmid library was maintained in *E. coli* cells by transfection of *E. coli* DH5α with the recombinant phage virus particles. A total of 3,168 cosmid clones were individually preserved in 96-well microtiter plates at −80°C. The pAF1 vector and the library construction have been described previously (49, 51).

Screening of genomic DNA libraries. The *A. parasiticus* lambda genomic DNA library (approximately 2×10^5 PFU/ml) was screened by using the *omtA* gene cDNA fragment as the probe (1.16-kb *Eco*RI-*Eco*RI cDNA fragment from the 1.46-kb *Eco*RI-*Xho*I full-length *omtA* gene cDNA clone [62]). The *A. parasiticus* cosmid library was screened by colony hybridization using DNA fragments internal to the *nor-1* gene (0.7-kb *Xho*I-*Xho*I cDNA fragment), *aflR* gene (1.9-kb *Bam*HI-*Bam*HI genomic DNA fragment), *ver-1* gene (1.0-kb *Eco*RI-*Xho*I cDNA fragment), or *omtA* gene (1.16-kb *Eco*RI-*Eco*RI and 0.3-kb *Eco*RI-*Xho*I cDNA fragments from the 1.46-kb *Eco*RI-*Xho*I insert from *A. parasiticus* SRRC 143) as probes. The *A. flavus* cosmid genomic DNA library was screened by hybridization to the master membrane plate and then to each of the membrane plates corresponding to the positive pools on the master plate by using the same gene fragments internal to the *nor-1*, *ver-1*, *aflR*, and *omtA* genes used above as probes.

Restriction mapping of positive clones and Southern hybridization conditions. Each individual lambda or cosmid clone was digested with all combinations of the four restriction enzymes, *Eco*RI, *Bam*HI, *Sal*I, and *Hin*dIII and separated on a 0.8% agarose gel. The DNA was transferred onto a GeneScreen Plus membrane (NEN Research Products, Boston, Mass.) by the semidry blotting method. The multiple membranes were probed with radioactive [α-^{32}P]dCTP-labelled *Bam*HI or *Eco*RI fragments from each individual clone by the random primer DNA labelling method (random-primed DNA labelling kit; Pharmacia Biotech Inc., Piscataway, N.J.). The restriction maps of the representative clones were based on the overlapping region and the resulting hybridization pattern. Hybridization was conducted in a solution containing 50% formamide, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) at 42°C overnight and was followed by two washes with a hybridization washing solution (2× SSPE and 0.1% SDS) at 60°C for 30 min each. A molecular weight standard (1-kb marker ranging from 0.1 to 12 kb) purchased from GIBCO-BRL was used in Southern blots as a size reference.

Isolation of total RNA. To find potential aflatoxin biosynthetic pathway-related transcripts from *A. parasiticus* that were neighboring the *omtA* gene, *A. parasiticus* SRRC 143 mycelia grown for 18, 24, 48, 72, or 96 h in A & M medium (a medium that induces aflatoxin B_1 production) were harvested and then pulverized to a fine powder in the presence of liquid nitrogen in a Waring blender. Total RNA was isolated from the mycelia by the hot phenol purification method (3).

Northern (RNA) blot analysis. A total of 10 μg of RNA per lane was separated on a 1.2% agarose gel containing 1.5% formaldehyde. The RNA was capillary transferred to a GeneScreen Plus membrane (NEN Research Products). To identify transcripts potentially involved in aflatoxin biosynthesis in the neighboring region of the *omtA* gene, RNA blots were probed with [α-^{32}P]dCTP-labelled 4.9- and 2.4-kb *Bam*HI fragments, respectively, located next to the *omtA* gene in *A. parasiticus* SRRC 143. Low-range RNA molecular weight standards (ranging from 0.16 to 1.77 kb) purchased from GIBCO-BRL were used.

Construction and screening of the cDNA library. Poly(A)$^+$ RNA from *A. parasiticus* SRRC 143 was separated from the total RNA by an oligo(dT)$_n$ column. A cDNA library was constructed from the poly(A)$^+$ RNA by using the Uni-ZAP XR vector (Stratagene). To identify potential aflatoxin pathway genes neighboring the *omtA* gene, the cDNA library (approximately 2×10^5 PFU/ml) was screened with [α-^{32}P]dCTP-labelled 4.9- and 2.4-kb *Bam*HI fragments as used in the Northern blot according to the instruction manual from Stratagene.

Nucleotide sequence accession numbers. The *ord-1* and *ord-2* cDNA nucleotide sequence data from wild-type *A. parasiticus* SRRC 143 have been submitted to GenBank. The assigned accession number for *ord-1* is L40839, and that for *ord-2* is L40840.

RESULTS

Transcripts neighboring the *omtA* gene detected. To detect transcripts neighboring the *omtA* gene potentially involved in the aflatoxin biosynthetic pathway, total RNA was probed with 4.9- and 2.4-kb *Bam*HI fragments adjacent to the *omtA* gene. Two transcripts accumulated in the mycelia after 24-h growth (data not shown). Two cDNA clones, designated *ord-1* and *ord-2*, corresponding to the transcripts, were obtained by using the same 4.9- and 2.4-kb *Bam*HI fragments as those in the probes to screen the cDNA library. The newly identified cDNA clones were sequenced (Fig. 2). The 1,284-bp *ord-1* cDNA contains an open reading frame encoding 396 amino acids (calculated molecular mass, 45 kDa) (Fig. 2A). A search in the GenBank and EMBL databases for possible homology to other known enzymes showed that *ord-1* contains two highly conserved motifs corresponding to over 50 cytochrome P-450-type enzymes including monooxygenases and dehydrogenases (12, 47, 50, 61). The cysteine residue within the second motif is believed to provide a ligand for heme binding (47, 50, 61). However, *ord-1* does not demonstrate much homology with a recently reported gene sequence from *A. nidulans* which also demonstrates over 30% nucleotide homology to cytochrome P-450 monooxygenases (39). The 1,033-bp *ord-2* cDNA contains an open reading frame encoding 286 amino acids (calculated molecular mass, 30.6 kDa) (Fig. 2B). A search in the GenBank and EMBL databases failed to identify any gene significantly homologous to the *ord-2* cDNA sequence and the deduced amino acid sequence. When cDNA inserts were used as probes to perform Southern hybridizations with the lambda and cosmid genomic DNA clones, *ord-1* hybridized to 3.1- and 8-kb *Eco*RI fragments in *A. parasiticus* and a 6.1-kb *Eco*RI fragment in *A. flavus*; *ord-2* hybridized to an 8-kb *Eco*RI fragment in *A. parasiticus* and a 4.4-kb *Eco*RI fragment in *A. flavus.*

Restriction endonuclease analysis and mapping of the positive clones. Ten *omtA*-positive *A. parasiticus* lambda clones, 15 both *ver-1*- and *omtA*-positive *A. parasiticus* cosmid clones, and 5 *nor-1*- or *aflR*-, *ver-1*-, and *omtA*-positive *A. flavus* cosmid clones containing two or more of the *nor-1*, *aflR*, *ver-1*, *omtA*, *ord-1*, and *ord-2* genes were identified and characterized by restriction enzyme analysis. Representative clones sharing overlapping regions were further analyzed by restriction mapping. To determine the location of each gene, additional DNA fragments that were internal to each of the genes *pksA*, *nor-1*, *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA* were used as probes. The restriction maps were based on the overlapping regions and the resulting hybridization pattern. For *A. parasiticus*, the representative overlapping clones are shown in Fig. 3. Lambda clone 1 contained *ord-1*, *ord-2*, and *omtA*, whereas clone 6 contained only *omtA*. Cosmid clones 2 and 4 contained *ord-2*, *omtA*, and part of *ord-1*; cosmid clones 1, 3, and 12 contained *ver-1*, *ord-1*, *ord-2*, and *omtA*; and cosmid clone 6 contained *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA*. Cosmid clone 7 was identical to the NorA cosmid (19), which contained *pksA*, *nor-1*, *aflR*, and *ver-1* (previously named *ver-1A* [42a]). Four restriction enzymes, *Eco*RI, *Bam*HI, *Sal*I, and *Hin*dIII were used for the construction and confirmation of the restriction patterns of the overlapping clones. For simplicity, only the *Eco*RI restriction pattern of the overlapping clones is presented in Fig. 3. For *A. flavus*, 3,168 cosmid clones were screened with the probes used

A

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-44                    CCAGTCCTATCTACACGAGTAATCCGGAGTTTCCCAAGGACCCG
  1 ATGCATCTTCCGCCCTTCCACAATGGAACCCCAGGCATCCTGGCCGCCGACCATGCACAC
  1  M  H  L  P  P  F  H  N  G  T  P  G  I  L  A  A  D  H  A  H
 61 CACCGCCGCTATCGACGACTGTTGGCCTTTTCATTCTCCGACAAAGGTTTGCGGCACGAG
 21  H  R  R  Y  R  R  L  L  A  F  S  F  S  D  K  G  L  R  H  E
121 CGCAGTCTAATCGAACGCAGTATTGACCTGCTCATCACGCAGCTCCATGAGAACTGTGGC
 41  R  S  L  I  E  R  S  I  D  L  L  I  T  Q  L  H  E  N  C  G
181 CAGGGCTCTGGACTCGCGCTGTGGTTCAACTGGGCCACCTTCGACATTATCGGCGACCTC
 61  Q  G  S  G  L  A  L  W  F  N  W  A  T  F  D  I  I  G  D  L
241 GCCTTTGGAGACTCATTCGGCTGCCTGGAGAACGTCCAGACGCATCCATGGATTGCATCC
 81  A  F  G  D  S  F  G  C  L  E  N  V  Q  T  H  P  W  I  A  S
301 ATTCAGGGTAATGTCAAGTTGATTCCCATTCTGAACGCCTTTCGTCGCTACCGACTGGAT
101  I  Q  G  N  V  K  L  I  P  I  L  N  A  F  R  R  Y  R  L  D
361 GGTCTTCTGCGGCTTCTGGGATCCCGCAAGCTGTTAGAACAGCGACGCCGGAACGCACAG
121  G  L  L  R  L  L  G  S  R  K  L  L  E  Q  R  R  R  N  A  Q
421 TTCACGACCGACCAGGTCGACCGTCGTCTCAAAAACTCCAGCACCCCACGCGGCGATATC
141  F  T  T  D  Q  V  D  R  R  L  K  N  S  S  T  P  R  G  D  I
481 TGGGATGCTGTACTGGCCCAGAAGCCCCACGGGGAACCGCCCATGACGCGGGATGAAATG
161  W  D  A  V  L  A  Q  K  P  D  G  E  P  P  M  T  R  D  E  M
541 ATCAGTAATGCCAGTGCCATCGTGCTGGCTGGTAGCGAGACTTCGGCCACCTTGTTAAGT
181  I  S  N  A  S  A  I  V  L  A  G  S  E  T  S  A  T  L  L  S
601 ATTTGCACTTGGCTGTTGCTAAAGAACCCGAGCCATCTGCACCAGCTTACCTCTCGGATA
201  I  C  T  W  L  L  L  K  N  P  S  H  L  H  Q  L  T  S  R  I
661 CGATCGCAGTTCACCCACGCATCGGAGATTGACTCCCAGAGTGTCTCGCGTGTCGAGGGA
221  R  S  Q  F  T  H  A  S  E  I  D  S  Q  S  V  S  R  V  E  G
721 CTGCAGGCAGTTCTGGAGGAATCACTGCGTCTGTATCCACCGGTACCCATGCAAAGTAAC
241  L  Q  A  V  L  E  E  S  L  R  L  Y  P  P  V  P  M  Q  S  N
781 CGCATCGTGCCCCAGGCGGGGGCTTATATCCGCAGGAGGATGGGTGCCCGGGGGCACATCA
261  R  I  V  P  Q  A  G  A  Y  I  A  G  G  W  V  P  G  G  T  S
841 GTCGGCCTACAACAGTTCGTCGCCTGCAGGTCATCCAGTAACTTCCATCGTCCCGACGAA
281  V  G  L  Q  Q  F  V  A  C  R  S  S  S  N  F  H  R  P  D  E
901 TTCCTGCCTGAGCGTTGGCAAGGCCAGGGTGAATTCGCCCATGATCGCCGCGAGGTATCA
301  F  L  P  E  R  W  Q  G  Q  G  E  F  A  H  D  R  R  E  V  S
961 CAACCGTTCAGCATCGGACCGCGGAATTGCATTGGACGACAGCTAGCCTACGTGGAAATG
321  Q  P  F  S  I  G  P  R  N  C  I  G  R  Q  L  A  Y  V  E  M
1021 CGGTTGATCCTAGTCAAGCTTCTCTGGCATTTCGACCTACGATTAGATACTACGCGGATG
341  R  L  I  L  V  K  L  L  W  H  F  D  L  R  L  D  T  T  R  M
1081 AAGGATACAGACTGGCTGGCAGAGCAGGGCATCTGGATTCTGTGGGATAAGAACCGTTGT
361  K  D  T  D  W  L  A  E  Q  G  I  W  I  L  W  D  K  N  R  C
1141 GGGTTACGCTGGAACCTCGCTATGAGTAGTAGCTCCAACTATTCTCTTTAGGCATACAGT
381  G  L  R  W  N  L  A  M  S  S  S  S  N  Y  S  L  *
1201 CATAGAGTCTCATAATTTACGTAAAAAAAAAAAAAAAAA
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B

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-95                              AAGCTTGCACGGGTAGGCGTGGTAAGCTCACTATA
-60 AAGCTGGATTGACTCCCTCCTAGGTGTGCATCTCTCATTCCTGCTCTGTCTTATAGCAAT
  1 ATGGTCACATACGCCCTCCTCGGGGCCACTGGGGCCACTGGCTCCAGTATTCTACGTCAT
  1  M  V  T  Y  A  L  L  G  A  T  G  A  T  G  S  S  I  L  R  H
 61 CTCCTTCAAAAGGGCCCGACTCTCTTCACATTCAAGTACTCGTTACATCGAAAGTGAAG
 21  L  L  Q  K  G  P  D  S  L  H  I  Q  V  L  V  T  S  K  V  K
121 TTACTACAAGCGTTCCCAGATCTCGAGACGACAAGACGCCCACAAGTTCACGTTATACAA
 41  L  L  Q  A  F  P  D  L  E  T  T  R  R  P  Q  V  H  V  I  Q
181 GGAATGTCTACAGACTCTGATGCTTTGAGTGAGTGCCTCAGGAATGCTAGTATTGTTTTC
 61  G  M  S  T  D  S  D  A  L  S  E  C  L  R  N  A  S  I  V  F
241 ATGTGTGTGGCGCAAAATGGGAGCCCAATTGGCACTACATTGTGCCAGGATAGTGCCCGC
 81  M  C  V  A  Q  N  G  S  P  I  G  T  T  L  C  Q  D  S  A  R
301 TCCATCATATCCGTGCTCCATCAACAACACCAGTCAGAGGGAGCAAGTTACCAGCCATGC
101  S  I  I  S  V  L  H  Q  Q  H  Q  S  E  G  A  S  Y  Q  P  C
361 ACAATCGTTCAACTTCGCTCAGCCTCTTTGAACCCTGCACTGGCGGCCCAGGTGCCGGCC
121  T  I  V  Q  L  R  S  A  S  L  N  P  A  L  A  A  Q  V  P  A
421 TTCGTTCATCGAATAGTCAGCTTCTGCCTCTTCGGCAACTATGCGGATATCAAGCAAGCG
141  F  V  H  R  I  V  S  F  C  L  F  G  N  Y  A  D  I  K  Q  A
481 TGCCAATACTACAGTGAAGCACAGAAGCAAGGGACATTGGAATACATTCTCGTGGACCCT
161  C  Q  Y  Y  S  E  A  Q  K  Q  G  T  L  E  Y  I  L  V  D  P
541 CCTACGCTTCATGATGCGAATGGAACACATCCAACGGGCTACAGACTAATCTCCACGGAG
181  P  T  L  H  D  A  N  G  T  H  P  T  G  Y  R  L  I  S  T  E
601 CCCCAAGCTACTGCCCTCAGCTACGCTGACTTAGGAGCTGCGATGTGTGAGATTGCTCAT
201  P  Q  A  T  A  L  S  Y  A  D  L  G  A  A  M  C  E  I  A  H
661 CGCGAAAGAGTTCCACGGGCGCGCGGTGGGTGTAACTGCCACTGGTCGTGTCCGTCAAAC
221  R  E  R  V  P  R  A  R  G  G  C  N  C  H  W  S  C  P  S  N
721 CTGGGGAGTCCTACTGCGTCATCTTTTGGAAGGTGGATCGTCTCGTTTAAGGGAGACAAT
241  L  G  S  P  T  A  S  S  F  G  R  W  I  V  S  F  K  G  D  N
781 AGCGAAGGAAGCAGTGGTTGTCAGGGTGTTGTGTATTTTCTTGGTGATATTGGCGTGTCT
261  S  E  G  S  S  G  C  Q  G  V  V  Y  F  L  G  D  I  G  V  S
841 GATGTCCTCATTGTAGGTTAAACTAAGGGCATATCAATTCTCCTGCCATGAAAGCTTCAT
281  D  V  L  I  V  G  *
901 GCTACGCTGTATTGAAAACTGTGGAAAAACGGTCGCCGAGAGAATGCGAGGCTGTTATAA
931 AAAAAAAA
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FIG. 2. Nucleotide and deduced amino acid sequences of the putative *ord-1* and *ord-2* cDNA clones in *A. parasiticus*. The full-length 1,284-kb cDNA sequence of the *ord-1* gene containing an open reading frame of 1,188 bp encoding 396 amino acids (A) and the *ord-2* gene containing an open reading frame of 858 bp encoding 286 amino acids (B) were determined. Nucleotides (upper sequence) and amino acid residues (lower sequence) are numbered on the left. The conserved regions for P-450-type enzymes are underlined, and the conserved amino acids are boldfaced. The stop codon is indicated (*). Note that the *ord-2* cDNA sequence is truncated, missing 5 nucleotides from its 5′ end. The 5′ sequence is determined by genomic sequence data.

above. Five *aflR*- or *nor-1*-positive clones were identified, and four of them were further characterized. Clones 8B9 and 20B11 contained *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA* genes; clone 30E1 contained *nor-1*, *aflR*, and *ver-1* genes; and clone 5E6 contained *nor-1* gene. The four representative overlapping clones are shown in Fig. 3.

Organization and arrangement of the aflatoxin pathway genes. The cloned genes *nor-1*, *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA* were mapped in *A. parasiticus* and *A. flavus*, whereas the recently cloned genes *pksA*, *uvm8*, and *aad* were mapped only in *A. parasiticus*. In *A. parasiticus*, two lambda clones and seven cosmid clones were aligned to each other on the basis of the overlapping regions and the aflatoxin pathway genes they contain. The *pksA* gene is located on the very left end immediately next to the *nor-1* gene. The *uvm8* gene is located between the *nor-1* and *aflR* genes. The *aad* gene is located between the *aflR* and *ver-1* genes immediately next to the *ver-1* gene. The *ord-1* and *ord-2* genes are located between the *ver-1* and *omtA* genes (Fig. 3). The distance between *nor-1* and *aflR* is 14 kb; that between *aflR* and *ver-1* is 7 kb, and that between *ver-1* and *omtA* is 10 kb (Fig. 3). In *A. flavus*, four cosmid clones were aligned to each other on the basis of the overlapping regions and the aflatoxin pathway genes they contain. The distance between *nor-1* and *aflR* is 14 kb; that between *aflR* and *ver-1* is 9 kb, and that between *ver-1* and *omtA* is 8 kb (Fig. 3). The order of these genes on the chromosome in *A. flavus* is the same as that in *A. parasiticus.*

By comparison of the overlapping regions of all the genomic clones from *A. parasiticus* and *A. flavus*, a physical map of all the known aflatoxin pathway genes was constructed (Fig. 3). All of the identified genes related to aflatoxin biosynthesis are located within a 60-kb DNA region. The order of these genes on the chromosome in *A. parasiticus* is *pksA*, *nor-1*, *uvm8*, *aflR*, *aad*, *ver-1*, *ord-1*, *ord-2*, *omtA*, and the order in *A. flavus* is *nor-1*, *aflR*, *ver-1*, *ord-1*, *ord-2*, *omtA*. In addition, the direction of transcription of each gene was determined by comparison of detailed restriction digest maps (with four enzymes) of genomic DNA and cDNA sequences of these genes and the deduced amino acids of cDNA. The *nor-1*, *aad*, and *ver-1* genes are transcribed from left to right, while the *pksA*, *uvm8*, *aflR*, *ord-1*, *ord-2*, and *omtA* genes are transcribed in a direction opposite to that of the *nor-1*, *aad*, and *ver-1* genes, i.e., from right to left in *A. parasiticus* (Fig. 3). The direction of transcription in *A. flavus* and *A. parasiticus* is the same for the identified genes. The distances between any two of the *nor-1*, *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA* genes are similar in the two species.

DISCUSSION

The organization of aflatoxin pathway genes in the toxin-producing fungi *A. parasiticus* and *A. flavus* is similar to that of the genes for other microbial secondary metabolites, in which the pathway genes are clustered, e.g., genes encoding penicillin or cephalosporin biosynthetic pathway enzymes (2, 26, 28, 30, 43, 44, 54, 55). The antibiotics penicillin and cephalosporin are secondary metabolites produced not only by prokaryotic bacteria but also by eukaryotic fungi, and the genes (*pcbAB*, *pcbC*, and *penDE*) coding for the biosynthetic enzymes for these antibiotics in the gram-positive bacterium *Nocardia lactamdurans* (26) and gram-negative bacterium *Flavobacterium* sp. 12154 (54) are clustered. More importantly, the penicillin biosynthetic genes in the eukaryotic fungi *Penicillium chrysogenum* (28, 55), *Cephalosporium acremonium* (30), and especially *A. nidulans* (*acvA*/*pcbAB*, *pcbC*, and *penDE* genes) (43, 44), a species taxonomically related to the two aflatoxin-producing fungi *A. parasiticus* and *A. flavus*, are all clustered as well. Recent data have also shown that the biosynthetic pathway genes of trichothecene, a non-polyketide-derived fungal myco-

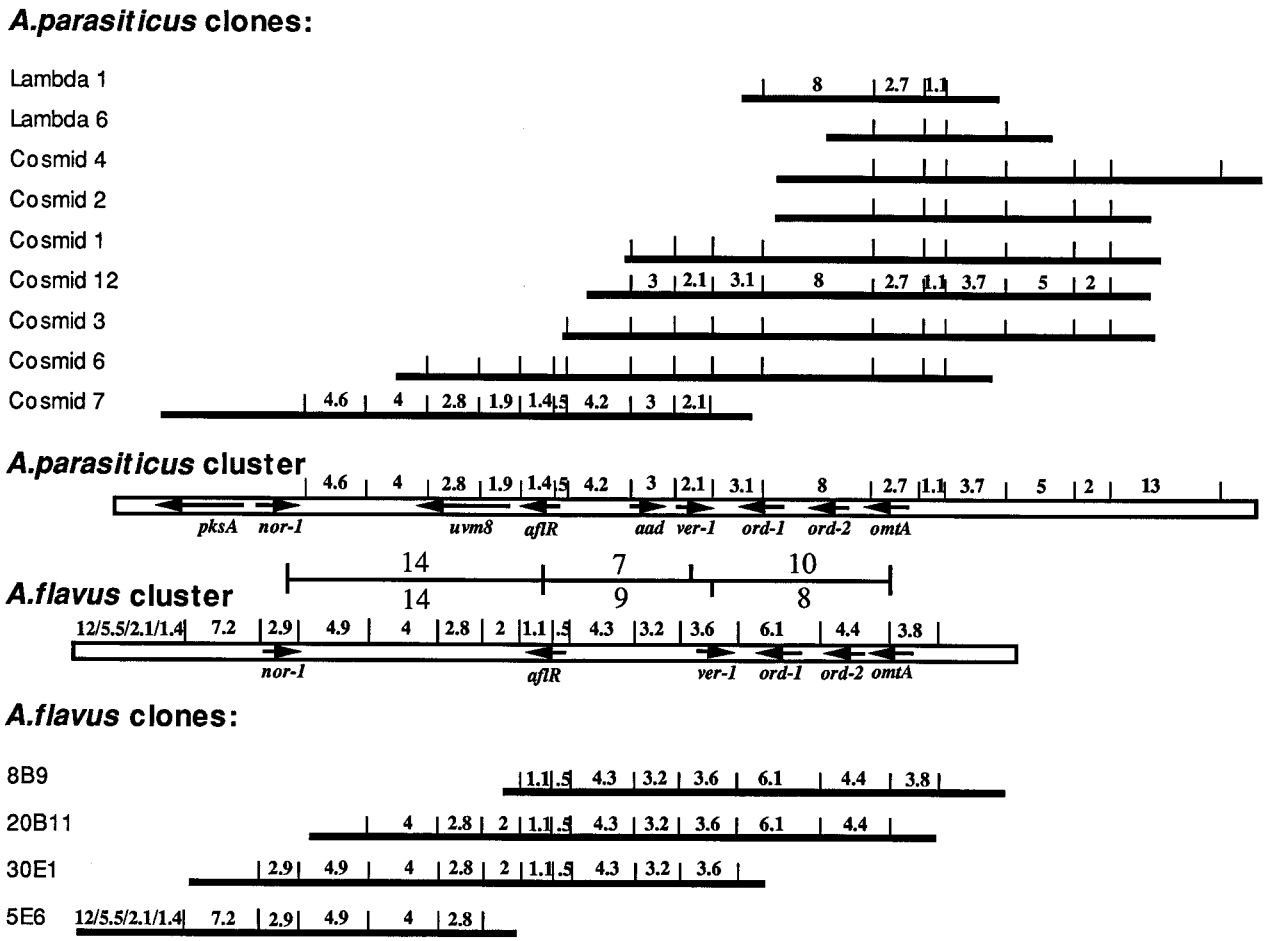

FIG. 3. *Eco*RI restriction pattern of the *A. parasiticus* and *A. flavus* overlapping genomic DNA clones and linear arrangement of aflatoxin pathway genes on the chromosome. The clustered aflatoxin biosynthetic pathway structural and regulatory genes in *A. parasiticus* and *A. flavus* are shown. The cluster map was derived from the restriction map overlapping genomic DNA clones from *A. parasiticus* (top) and *A. flavus* (bottom). The approximate sizes and the directions of transcription of the identified genes are indicated (arrows). Numbers indicate the distances in kilobase pairs between restriction sites and between major genes, assuming that each gene occupies 2 kbp.

toxin, from *Fusarium sporotrichiodes* were also clustered (32). These observations may suggest that the genes coding for secondary metabolites in fungal systems tend to be organized in clusters. The significance of the tight cluster of aflatoxin-biosynthetic genes is not known. No evidence has been obtained to suggest that the presence of the genes organized in a cluster confers any selective advantage for the survival of the organism since aflatoxins do not deter the growth of competing organisms and also do not increase the ability of the organism to invade its hosts. However, the cluster of aflatoxin pathway genes may allow all of the pathway genes to be expressed rapidly upon onset of secondary metabolism since aflatoxins start to accumulate rapidly after 18 to 20 h of mycelium growth. This may ensure that all of the aflatoxin pathway enzymes are available at the same time for efficient production, assuming that the fungus needs to produce aflatoxins at a rapid rate. There is also no evidence that the toxin gene cluster is the result of either horizontal gene transfer or vertical transmission from common ancestral genes since no pattern of homology is evident between the aflatoxin pathway genes and the sources of their homologs.

Earlier reports suggested that some of the aflatoxin pathway genes are linked in the aflatoxin-producing and non-aflatoxin-producing strains of *A. flavus* and *A. parasiticus* (36, 37). Parasexual studies identified four (48), six (10), and seven (40) linkage groups in *A. parasiticus*. Conflicting observations regarding the linkage groups of the genes responsible for aflatoxin biosynthesis were also presented, with *nor-1* and *ver-1* genes being found on the same linkage group (13, 16, 52) as well as on separate linkage groups (40). Data presented in this study clearly demonstrate that not only are *nor-1* and *ver-1* linked in both species but other aflatoxin pathway genes are also linked. All of the identified aflatoxin biosynthetic pathway genes are clustered in a relatively small (60-kb) region. The order and distance between these genes were determined by direct mapping on the cloned fragments, and it is unlikely that the gene organization we have identified is due to random association during library construction. The overall order and organization of the clusters from the two *Aspergillus* species are similar, but the spacing between the genes is not perfectly conserved.

Karyotyping studies have also confirmed that the aflatoxin pathway genes are clustered. Karyotype analysis of *A. parasiticus* confirmed that the *nor-1*, *ver-1*, and *omtA* were located on the same chromosome (56a). Studies of aflatoxigenic aspergilli indicate that these fungi have six to eight chromosomes ranging from 3 to >7 Mb (36); *A. nidulans*, the best characterized of these fungi, has six chromosomes (39). *verA* in *A. nidulans*,

the homolog of *ver-1* in *A. parasiticus*, is located on the left arm of chromosome IV (39). A similarity in gene arrangement between the two *Aspergillus* species can be expected.

Thus far, the order of most of the genes characterized in the gene cluster is coincident with the order of the enzyme activities in the aflatoxin biosynthetic pathway encoded by these genes. It is not clear whether any two or more of the aflatoxin pathway genes are cotranscribed, since Northern blot analysis of the *omtA* (62), *ord-1*, and *ord-2* genes (data not shown) showed no evidence of cotranscription even though these genes are closely linked and transcribed in the same direction (62). Studies on the expression and regulation of the aflatoxin biosynthetic regulatory and pathway genes involving *aflR*, *nor-1*, *ver-1*, and *omtA* showed that the transcription of *nor-1*, *ver-1*, and *omtA* is activated by the *aflR* gene product, AFLR. In other words, the transcription of the structural genes is dependent on the transcription of the *aflR* gene (18). The expression of the genes was turned on coordinately starting from *nor-1* to *ver-1* to *omtA*, even though the direction of the *omtA* gene transcription is opposite to that of the *nor-1* and *ver-1* genes. It may be possible that other aflatoxin pathway genes like *pksA*, *aad*, *ord-1*, and *ord-2* are also under AFLR regulation at the transcriptional level and that the transcription is turned on in a sequential order ensuring that the pathway is functioning efficiently. Preliminary evidence from this laboratory suggests that the regulatory *aflR* gene product, AFLR, turns on the transcription of each of the structural genes rather than the gene product of a structural gene turning on the transcription of the next structural gene in a sequential manner (18). The aflatoxin biosynthetic pathway, therefore, appears to be positively regulated in a manner similar to that of sulfur and nitrogen metabolism in filamentous fungi *A. nidulans* and *Neurospora crassa* (45). However, the biological significance, if any, of the observation that the order of the genes in the cluster is coincident with the order of pathway enzyme activities is unclear.

It appears that the sequences of the genes involved in aflatoxin biosynthesis are highly conserved both at the DNA and at the amino acid levels within the aflatoxigenic species of *A. parasiticus* and *A. flavus*. The DNA sequences of *omtA* from *A. parasiticus* SRRC 143 and *A. flavus* CRA01-2B were 97% homologous (63), and the sequences of the *aflR* genes were also very similar (95%) (16, 49, 56). In addition, the amino acid sequence of the *ver-1* gene in *A. parasiticus* SRRC 143 was 85% homologous to that of its counterpart, the *verA* gene, in the non-aflatoxin-producing species *A. nidulans* (39).

Data obtained in this study demonstrate that there may be two copies of *ver* and *aflR* in *A. parasiticus*. An additional *ver* gene-positive cosmid clone, designated Ver 8 (identical clones are Ver 10 and Ver 13), was identified among the *A. parasiticus* cosmid clones studied here. Ver 8 is identical to the cosmid clone Ver 3 identified earlier (52). Cosmid Ver 3 overlaps with cosmids Ver 2 and Ver 4 in the *ver-1* gene region (53a). The cosmid clone Ver 8 (Ver 3) differs from those presented in Fig. 3 with respect to the *ver* gene. Therefore, the *ver* gene on cosmid clones Ver 2, Ver 8 (Ver 3), and Ver 4 was tentatively named *ver-1B* (42a). The cosmid clone Ver 8 does not contain the *omtA* gene in a 30-kb region downstream of the *ver-1B* gene. Furthermore, the restriction patterns of the DNA flanking *ver-1B* are different from the *ver-1* (previously named *ver-1A* for the NorA cosmid [42a]) gene in Fig. 3. *ver-1B* is located in a 5.1-kb *Eco*RI fragment rather than in a 2.6-kb *Eco*RI fragment as in the clones shown in Fig. 3. Differences in genomic DNA homology between *ver-1* and *ver-1B* based on hybridization at higher stringency were observed. A duplicated region extending 12 kb upstream from *ver-1* that contains copies of the *ver* and the *aflR* genes has recently been identified (41a). Recent sequence data revealed that *ver-1B* is a nonfunctional pseudogene (42a).

Further research for identification and characterization of other aflatoxin pathway genes is under way. Additionally, a detailed characterization of the differences between the two aflatoxigenic species in gene distances and the duplication of a set of pathway genes only in *A. parasiticus* may explain the differences in toxin production between the two aflatoxigenic species. *A. parasiticus*, unlike *A. flavus*, produces the G toxins, aflatoxins G_1 and G_2; both species produce the B toxins, aflatoxins B_1 and B_2. Also, whereas few aflatoxin nonproducers are present in field strains of *A. parasiticus*, it is not uncommon for up to 45% of the field isolates of *A. flavus* to be non-aflatoxin producers (27, 31).

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