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Structural and Functional Analysis of the *nor-1* Gene Involved in the Biosynthesis of Aflatoxins by *Aspergillus parasiticus*

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The *nor-1* gene was cloned previously by complementation of a mutation (*nor-1*) in *Aspergillus parasiticus* SU-1 which blocked aflatoxin B1 biosynthesis, resulting in the accumulation of norsolorinic acid (NA). In this study, the nucleotide sequences of the cDNA and genomic DNA clones encompassing the coding region of the *nor-1* gene were determined. The transcription initiation and polyadenylation sites of *nor-1* were located by primer extension and RNase protection analyses and by comparison of the nucleotide sequences of the *nor-1* genomic and cDNA clones. A plasmid, pNA51-82, was created for one-step disruption of the *nor-1* gene by inserting a functional copy of the nitrate reductase (*niaD*) gene from *A. parasiticus* into the coding region of the *nor-1* gene. Transformation of *A. parasiticus* NR-3 (*niaD* Afl$^+$) with pNA51-82 resulted in *niaD*$^+$ transformants that accumulated NA and produced reduced levels of aflatoxin as determined by thin-layer chromatography and enzyme-linked immunosorbent assay analyses of extracts from mycelia and the growth medium. Southern analysis of genomic DNA isolated from the NA-accumulating transformants indicated that the wild-type *nor-1* gene in the chromosome had been replaced by the nonfunctional allele carried on pNA51-82. This recombinational inactivation event provides direct evidence that the *nor-1* gene is functionally involved in aflatoxin biosynthesis. Comparison of the predicted *nor-1* amino acid sequence with sequences in the GenBank and EMBL databases suggested that the protein is a member of the family of short-chain alcohol dehydrogenases, consistent with its proposed function as a keto reductase.

Aflatoxin is a secondary metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus*. The toxin has been shown to be a potent animal teratogen and carcinogen and is associated with liver disease in humans (10). Corn, cotton, peanuts, and other important crops can become contaminated with aflatoxin when infected by these filamentous fungi, resulting in contaminated foods that are staples in many areas of the world. Aflatoxin biosynthesis and aflatoxigenic fungi are receiving increased attention from plant pathologists, fungal geneticists, and crop breeders, who are developing a multifaceted approach to reduce or eliminate the toxin in food and feed. One likely application of these efforts will be the identification and specific disruption of several genes associated with aflatoxin production in a single strain. These genetically stable nonaflatoxigenic fungal strains can be tested for use as biological control agents. In support of this approach, naturally occurring nonaflatoxigenic strains of *A. flavus* and *A. parasiticus* have shown promise in biological exclusion of aflatoxigenic strains in greenhouse and field studies (16, 41). In addition, long-range studies on the regulation of the biosynthetic pathway can lead to direct approaches to the control of aflatoxin through genetic engineering of the host plant.

The biosynthesis of aflatoxin has been studied for many years by using mutants that are partially or fully blocked in aflatoxin production. The synthesis of aflatoxin B1 (AFB1), the most abundant and toxic of the aflatoxins, is thought to be essentially identical in *A. flavus* and *A. parasiticus* (3). The first apparent stable intermediate in the pathway is norsolorinic acid (NA), an anthraquinone, which is believed to be formed by a polyketide synthase from the condensation of one acetate and nine malonate subunits (5). NA is converted to averufin (AVF) via a multistep series of reactions. Yabe et al. (43) have demonstrated that the following sequence of reactions occurs in *A. parasiticus* NIAH26: NA to averantin (AVN) to hydroxyaverantin to AVF. On the basis of accumulation of pathway intermediates and feeding studies in mutants blocked in AFB1 biosynthesis, Bhatnagar et al. (5) have reviewed data supporting the presence of one or more of three alternative routes for conversion of NA to AVF: (i) NA to AVN to averufanin to AVF, (ii) NA to AVN to AVF (open form) to AVF, and (iii) NA to averufanin to AVF. The presence of alternative pathways could account for the fact that NA-accumulating strains created by UV or chemical mutagenesis still produce aflatoxin, although in reduced quantities (15). This observation could also be explained by the presence of two different enzyme activities capable of converting NA to the same subsequent pathway intermediate (i.e., AVN). The remaining steps in the pathway, as it is now understood, occur as follows: AVF to hydroxyversicolorone to versiconal hemiacetal acetate to versicolorin B to versicolorin A to demethylsterigmatocystin to sterigmatocystin to *O*-methylsterigmatocystin to AFB1 (5). Aflatoxin G1 (AFG1) is hypothesized to be derived from sterigmatocystin and *O*-methylsterigmatocystin (reviewed in reference 5).

Aflatoxin is a secondary metabolite, and its production usually begins during idiophase, upon the depletion of nutrients necessary for primary metabolism, although other conditions for induction exist. Regulatory mechanisms involved in the induction of aflatoxin biosynthesis are complex and not fully understood. The many studies on the compounds and conditions that induce or inhibit aflatoxin biosynthesis in culture have been reviewed previously (46). The pattern of induction suggests that carbon catabolite induction occurs (6, 7). To study the regulation of the AFB1 biosynthetic pathway

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at the molecular level, much effort has recently been focused on purifying enzymes involved in AFB1 synthesis and isolating the genes which encode them. Genes associated with aflatoxin biosynthesis have been cloned by complementation of blocked mutants of *A. parasiticus* and *A. flavus*. The *nor-1* gene (previously *nar-1* for NA related), encoding an activity associated with the conversion of NA to AVF, and the *ver-1* gene, encoding an activity associated with the conversion of versicolorin A to sterigmatocystin, were isolated in this way (9, 35). Another gene, *aflR* (formerly *afl-2*), from *A. flavus*, apparently involved in the regulation of the pathway, has also been isolated (29) along with its *A. parasiticus* homolog, *aflR* (formerly *apa-2*) (8). By using an alternative cloning strategy, differential screening of an *A. parasiticus* genomic DNA library was used to isolate genes whose expression coincides with aflatoxin production (17). Specific identification of the activities of these genes has not been reported.

Purification of enzymes involved in the biosynthesis of aflatoxin has been successful in only a few cases because of the apparently low quantities of the enzymes and their instability. For example, the purifications of a versiconal cyclase involved in dehydration of versiconal (23) and of a methyltransferase associated with the conversion of sterigmatocystin to *O*-methylsterigmatocystin have been reported (21). Of relevance to the work reported here, two different NA reductases associated with the conversion of NA to AVN (4, 11) have been purified, suggesting that *nor-1* may encode only one of multiple NA reductase activities.

The isolation of genes and proteins apparently involved in aflatoxin production marks a turning point in the study of the regulation of aflatoxin biosynthesis. For example, time course studies on the appearance of aflatoxin, enzymes related to aflatoxin production, and RNA transcripts of *nor-1* and *ver-1* indicate that several of the genes are probably regulated in part at the transcriptional level (11, 12, 36). To continue elucidating the molecular mechanisms which regulate AFB1 gene expression, it was vital to firmly establish the functional role of cloned genes in the AFB1 biosynthetic pathway. In this study, the physical structure of *nor-1* was determined by nucleotide sequence and transcript mapping analyses. Evidence for the specific disruption of *nor-1* and the effect of this disruption on aflatoxin biosynthesis in *A. parasiticus* (39) are also presented. These data confirm the previously hypothesized direct role of *nor-1* in aflatoxin biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions. Plasmids were amplified in *Escherichia coli* DH5α F′ᵉ [F′/*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)U169 (m80*lacZ*ΔM15)]. *A. parasiticus* NRRL 5862 (SU-1) served as the aflatoxin-producing wild-type strain. *A. parasiticus* NR-3 is a nitrate reductase-deficient strain derived from NRRL 5862 by spontaneous mutation to chlorate resistance (20) and was used as the host strain for *nor-1* gene disruption.

Fungal strains were maintained as frozen spore stocks (10^8 cells per ml) in 20% glycerol. Coconut agar medium (CAM) (1), an aflatoxin-inducing medium, was used for screening fungal strains for aflatoxin and NA accumulation by visualization of blue or orange fluorescence, respectively, under long-wave UV light (14, 22). YES broth (2% yeast extract, 20% sucrose [pH 5.5]), an aflatoxin-inducing medium, was used to grow mycelia for DNA preparations and for thin-layer chromatography (TLC) and enzyme-linked immunosorbent assay (ELISA) analyses. Reddy's medium (32), also aflatoxin inducing, was used to grow mycelia for RNA preparations.

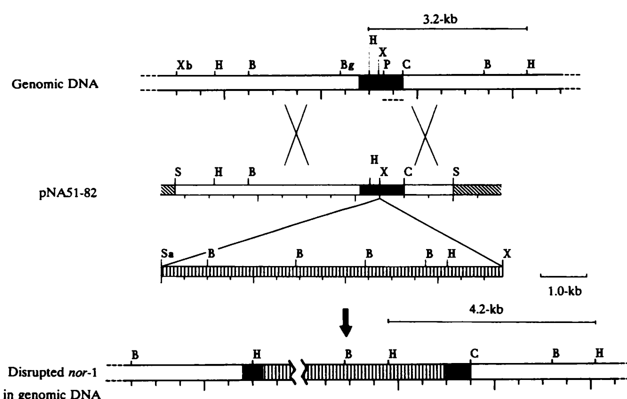

FIG. 1. Strategy for the disruption of *nor-1* from *A. parasiticus*. Restriction maps of the *nor-1* region of the chromosome (top), disruption vector pNA51-82 (middle), and proposed map of the *nor-1* region following the disruption event (bottom) are shown. Also indicated are the *nor-1*-transcribed region (solid bars), pUC19 vector (bars with diagonal lines), chromosomal regions containing the *niaD* gene (bars with vertical lines), and *nor-1* flanking regions (open bars). Positions and sizes of *Hin*dIII fragments seen in Southern analysis of genomic DNA of wild-type and disrupted transformants are indicated above corresponding regions of genomic DNA. The position of the riboprobe used for Southern analysis is indicated by a dashed line below the *nor-1* region. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; Sa, *Sal*I; S, *Sph*I; Xb, *Xba*I; X, *Xho*I.

Nucleotide sequence analysis. Nucleotide sequence analysis of genomic DNA was performed by the dideoxy chain termination method (33) with Sequenase II (U.S. Biochemical Corp., Cleveland, Ohio) as described in the manufacturer's instructions. Restriction fragments of the *nor-1* gene were cloned into pBluescriptSKII$^-$ (Stratagene Cloning Systems, La Jolla, Calif.) to generate plasmids pSK05 (450-bp *Bgl*II-*Hin*dIII), pSK03 (330-bp *Hin*dIII-*Pst*I), and pSK07 (720-bp *Pst*I-*Cla*I) (Fig. 1). Cloned inserts were sequenced on both strands with the T7 and T3 primers supplied in the kit. From this initial sequence information, three oligonucleotide primers (15 nucleotides each) were used to confirm the sequence and to remove ambiguities (Fig. 2). Plasmid pNA17, constructed by ligation of the 1.5-kb *Bgl*II-*Cla*I fragment (Fig. 1) into *Bam*HI-*Cla*I-cut pBluescriptSKII$^-$ was used as a template for sequencing with the synthesized primers.

The *nor-1* cDNA was isolated by in situ plaque hybridization of an *A. parasiticus* cDNA library (35) with a radiolabelled *nor-1* probe. Sequence analysis of the *nor-1* cDNA fragment was performed with a TAQence sequencing kit (U.S. Biochemical Corp.), which uses the thermostable Δ*Taq* DNA polymerase.

Computer analysis of sequence data. Computer analyses of nucleotide sequence data were performed by using the Wisconsin Genetics Computer Group package. The locations 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 *Aspergillus nidulans* genes reported by Lloyd and Sharp (25). The GenBank accession number for *A. parasiticus nor-1* is L278801.

Nucleic acid isolation and analysis. DNA was purified from *A. parasiticus* by a published modification (37) of a phenol-chloroform protocol developed for mammalian DNA (2). Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals or New England BioLabs and used as

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   1  TCTAGAACTAGTGGATCTCTGCTATTAAGTCGGTGATTAGCGTGCTGGATGCGCAATATCATTAGATATGCCTTTTCTTTCTTTTCTTCTTTAATTTTCT  100

 101  TTTGGGGGGGCCAGCACGGAGATCGAATTTGTTGGCATACCATCAAATGCCTGCCACCCAACCGCCCAACTCGGCCAGCGACCAACACACCGCCATATAT  200

 201  AGTGGGATACGATCATGGGTCTTTGGTGGTTTCAACATTTCTTGAGTACTTTTCTAAGCCGTGACATAATGAACGGATCACTTAGCCAGCACGATCAAGA  300
  67                                 "  "        "                        M  N ~G  S  L  S  Q  H  D  Q  E   100

 301  GAGGCTCTCTACGCCATACCGGGATGGACCGCCTGAGGAGACGGTGTATTTGGTCACCGGGGCCAGCCGAGgtacggtctatcgaatcaggggtcctac  400
 101   R  L  S  T  P  Y  R  D  G  P  P  E  E  T  V  Y  L  V  T  G  A  S  A  G                               134

 401  gaaatttactcatcaaacgcagGCATCGGACGAGGTCTCATTGAAGCTTTTCTCCAACGTCCCAAAAGCACCGTGGTCGCTTGGCTGCGCAACGTCCGC  500
 134                         I  G  R  G  L  I  E  A  F  L  Q  R  P  K  S  T  V  V  A  W  L  R  N  V  R    167

 501  ACCGCTACGCCGGCACTCTCGGCACTAACCGTCGCTGAAGGCAGTCGGATGATCATAGTGCAGCTTAATTCCGACTCGGAAACTGATGCCCAGGCAGCCG  600
 167  T  A  T  P  A  L  S  A  L  T  V  A  E  G  S  R  M  I  I  V  Q  L  N  S  D  S  E  T  D  A  Q  A  A  V   200

 601  TCCAGACATTGCGGGAGGAGCACGGGGTGACGCACTTGGACGTGGTGGTTGCCAATGCGGCCATGGCGACGAACTTTGGGCCCGCGTCCACCATGCCCCT  700
 201   Q  T  L  R  E  E  H  G  V  T  H  L  D  V  V  V  A  N  A  A  M  A  T  N  F  G  P  A  S  T  M  P  L    234

 701  CGAGCATCTCCAGGCGCACATGATGGTCAACATGTATGCTCCCGTCCTACTGTTTCAGGCAACCCGCCTGATGCTGCAGCAGTCCAAGCAACAGGCCAAG  800
 234   E  H  L  Q  A  H  M  M  V  N  M  Y  A  P  V  L  L  F  Q  A  T  R  L  M  L  Q  Q  S  K  Q  Q  A  K    267

 801  TTTGTCTTGATCGGCGCCCCGATCAGCACCATCACCAACATGCACGACTACTCGCGGGCCCCACTGACGGCGTACGGAGTGTCGAAGCTGGCGGCCAACT  900
 267  F  V  L  I  G  A  P  I  S  T  I  T  N  M  H  D  Y  S  R  A  P  L  T  A  Y  G  V  S  K  L  A  A  N  Y   300

 901  ACATGGTGCGCAAGTTCCACTTTGAGAACAAATGGCTCACGGCCTTCATCATCGATCCAGGgtaatcacagtccgagggttcttgtcgcaacgtatattc  1000
 301   M  V  R  K  F  H  F  E  N  K  W  L  T  A  F  I  I  D  P  G                                          334

1001  gctgaccgaaacatgctagACATGTGCAAACTGATATGGGCGACCAAGGAGCGCGGCTGATGGGCCGTCCGCAGGCCCCAACAACTGTCGCAGACAGTGT  1100
 334                     H  V  Q  T  D  M  G  D  Q  G  A  R  L  M  G  R  P  Q  A  P  T  T  V  A  D  S  V   367

1101  GGCAGGCATCTGTGCTCGGgtatgaccattgccttcttctttgcgcgcgcagggagcgacgtgggctaacgttggggtggtagATTGATGAAGCGACCAA  1200
 367   A  G  I  C  A  R                                                              I  D  E  A  T  K    400

1201  GGAGACTACATCGGGGCACTTCGTTATCCACACGGATGGATCTCAACTCCCCTGGTAGGACGCTAGTGACGACGAAGCGCAGTCATATGTTTCTGAGACG  1300
 401   E  T  T  S  G  H  F  V  I  H  T  D  G  S  Q  L  P  W  *                                              434

1301  AGCAGAAACGTGCTGGCGTGGAGCTGGTCCAGAAAGGCGCGGTGAGGTCCTGGGTTCGTGTCGCGGCGGCTTTCGCTCGATCAGTTCGTGTATGACCTTT  1400

1401  TCGGTCTTTTCCGTTCCGTCTGTTTAGGCTCTCACAAGATAAAACCAAATTGAAACCTAACGTTCGTTTTCATGGACCCCTGATGGAAATATCGATACCG  1500
                                                                               ^
1501  TCG  1503
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FIG. 2. Nucleotide sequence of the 1.5-kb *Bgl*II-*Cla*I restriction fragment containing the *nor-1* gene. The predicted amino acid sequence is shown below the corresponding codon. Probable consensus sequences and other sites are indicated as follows: double underline, Hogness box; quotation marks, transcription initiation sites (predicted by primer extension); caret, polyadenylation site (predicted by cDNA sequence and confirmed by RNase protection); ~, 5′ end of cDNA; *, termination codon. Primers used in sequencing are underlined; the primer covering nucleotides 1122 to 1136 was antisense to the sequence indicated. Intervening sequences are in lowercase letters.

described in the manufacturer's instructions. Total RNA was purified from aflatoxin-induced cultures by using a hot phenol protocol described previously (27). Northern (RNA) and Southern analyses were performed by using published procedures (2) with a modified hybridization buffer and conditions recommended by Stratagene Cloning Systems. After the final wash, hybridization membranes were placed on X-ray film (Kodak XAR5) at −81°C for the times indicated in the figure legends.

RNase protection assay and primer extension analysis. The location of the 5′ end of the *nor-1* transcript was determined by primer extension analysis (2) using a 20-bp oligonucleotide primer complementary to the sense strand from nucleotide 283 to 302 (Fig. 2). The primer was hybridized to total cellular RNA (30 μg) from cultures of *A. parasiticus* NRRL 5862 grown in Reddy's medium and harvested 18, 48, or 60 h after inoculation or was hybridized to *Saccharomyces cerevisiae* tRNA (30 μg) as a control. As a size reference for the resulting extension fragments, nucleotide sequence analysis was conducted on pSK05 by using the same oligonucleotide primer and [α-^{32}P]dATP as the label. The resulting sequencing reactions were resolved alongside the extension reactions on a 6% denaturing polyacrylamide sequencing gel (2). The 3′ end of the *nor-1* transcript was localized by an RNase protection assay using RPAII, an RNase protection assay kit (Ambion, Inc., Austin, Tex.). Riboprobes were transcribed from the T7 promoter of pSK07 linearized with *Nde*I (ending at position 1284 [Fig. 2]) and *Pst*I (ending at position 779 [Fig. 2]). The riboprobes were hybridized overnight at 42°C to total cellular RNA (30 μg) from 60-h cultures described above or to torula yeast tRNA (30 μg). Protected fragments were separated by electrophoresis on a 6% denaturing polyacrylamide sequencing gel (2).

Plasmid construction and purification. A 5.1-kb *A. parasiticus* genomic DNA fragment encompassing the entire *nor-1* coding region plus 5′ and 3′ flanking regions (Fig. 1) was

subcloned from plasmid pNA51 (9) as an *Sph*I fragment into the *Sph*I site of pUC19, forming the plasmid pNA51-2. The disruption plasmid, pNA51-82, was constructed by insertion of the 8.2-kb *Xho*I-*Sal*I fragment of pSL82 (20), containing a functional copy of the nitrate reductase gene, *niaD*, into the *Xho*I site of pNA51-2 (Fig. 1). Plasmid minipreparations were performed by the boiling method (26); large-scale preparations were performed by the alkaline lysis procedure of Maniatis et al. (26).

Transformation of *A. parasiticus*. In separate experiments, circular or *Sph*I-cut pNA51-82 was used to transform *A. parasiticus* NR-3 to disrupt the *nor-1* gene. Polyethylene glycol-mediated transformation was carried out as described previously (28) with minor modifications (37). Nitrate-utilizing transformants were selected on Czapek-Dox agar (Difco) containing nitrate as the sole nitrogen source. Nitrate-utilizing transformants were transferred to CAM to screen for NA accumulation. Transformant clones were purified by isolating single spores three successive times.

Analysis of NA and aflatoxin production in *nor-1*-disrupted strains. AFB1 and intermediates in AFB1 biosynthesis which accumulated in aflatoxin-induced cultures of five *nor-1*-disrupted transformants and an NR-3 control were examined. Two hundred fifty-milliliter Erlenmeyer flasks containing 100 ml of YES broth were inoculated with 1.75×10^8 spores from pNA51-82 transformants or NR-3 and incubated without agitation at 30°C in the dark. Three flasks were prepared and analyzed for each strain. After 72 h of growth, mycelial mats were removed and blotted dry. One quarter (wet weight) of the mycelial mat was dried overnight at 60°C to determine dry weight. The remainder of each mycelial mat and the growth medium were extracted separately with 30 ml of acetone for 45 min with agitation. Subsequently, 50 ml of chloroform was added to the acetone mixtures, and extraction continued for 45 min more. The solvent portions of the extraction mixtures were collected and concentrated by evaporation. TLC analyses of the solvent extracts were performed on activated high-performance silica TLC plates (10 by 10 cm) in a chamber equilibrated with chloroform-acetone (95:5). Purified NA (generously provided by D. Bhatnagar, USDA Agricultural Research Service, New Orleans, La.) and AFB1 (Sigma Chemical Co., St. Louis, Mo.) were resolved on each plate as standards. The orange pigment that comigrated with the NA standard was scraped from the plates and dissolved in absolute ethanol, and the absorbance spectrum from 200 to 600 nm was determined.

Direct competitive ELISA analyses were performed on samples of the culture medium from the 72-h cultures to determine concentrations of AFB1. The procedure was performed as described by Pestka (31) with AFB1 monoclonal antibodies and AFB1-horseradish peroxidase conjugate (both kindly provided by J. Pestka, Michigan State University).

RESULTS

Nucleotide sequence analysis of *nor-1*. The nucleotide sequences of a *nor-1* genomic DNA clone (1.5-kb *Bgl*I-*Cla*I) and cDNA clone were determined (Fig. 2). Protein coding regions were predicted by use of the Wisconsin Genetics Computer Group software to test the randomness of codons and codon preference. The locations of intervening sequences were then identified by open reading frame analyses and confirmed by comparison of the genomic nucleotide sequence with the cDNA nucleotide sequence. The *nor-1* genomic DNA sequence is predicted to contain three introns (Fig. 2). Splice junctions were tentatively identified by comparing consensus sequences of intron boundaries and internal regions associated with lariat formation in *Aspergillus* spp. (18). A putative Hogness box (TATATA) was identified immediately upstream of the transcription initiation sites determined by primer extension (see below). The first ATG downstream from the proposed transcription initiation site begins at nucleotide 269 and marks the putative translation initiation codon. The TAG codon in the fourth exon appears to be the translation termination codon. It is located approximately 215 bp upstream of the polyadenylation site predicted by cDNA nucleotide sequence analysis and RNase protection assay (see below). This analysis results in a putative *nor-1* protein of 294 amino acids and a molecular mass of 29 kDa.

Analysis of transcripts from *nor-1*. The approximate location of the transcription initiation site of *nor-1* was determined by primer extension analysis. A 20-base oligonucleotide primer designed to anneal to nucleotides 283 to 302 in the genomic DNA sequence (Fig. 2) was hybridized to total cellular RNA purified from cells grown for 18, 48, and 60 h in Reddy's medium (AFB1 inducing). In preliminary experiments, at 18 h, the *nor-1* transcript was barely detectable by Northern analysis, whereas by 60 h, transcript accumulation reached a peak (data not shown). Two unique primer extension fragments, terminating at nucleotides 233 and 247, were visible in the 48- and 60-h samples (Fig. 3B), were barely visible in the 18-h samples, and were absent in the *S. cerevisiae* tRNA control lanes, suggesting that these extension products were derived directly from the *nor-1* transcript. Another extension fragment was faintly visible only in the 48- and 60-h samples and terminated at nucleotide 235 in the genomic DNA sequence (Fig. 2). Thus, there appear to be three (two major and one minor) transcriptional start sites within the 14-nucleotide region from nucleotides 233 to 247. In contrast, the cDNA nucleotide sequence began at nucleotide 275, downstream from the start site determined by primer extension, suggesting that the cDNA clone used in sequence analysis was truncated at the 5′ end.

The location of the transcription polyadenylation site was determined by RNase protection. Two riboprobes were transcribed from pSK07 cut with *Nde*I or *Pst*I and hybridized to total cellular RNA from *A. parasiticus* or to torula yeast tRNA. The sizes of three fragments protected by the riboprobe transcribed from the *Pst*I-linearized pSK05 (0.182, 0.100, and 0.288 kb) corresponded to the three exons (exon II [3′ of the *Pst*I site], exon III, and exon IV, respectively) and confirmed their positions in the gene. The fragment protected by the riboprobe transcribed from *Nde*I-linearized pSK05 (0.186 kb) corresponded to exon IV, 3′ of the *Nde*I site. The protected fragment corresponding to exon IV from both riboprobes localized the 3′ end of the transcript to between nucleotides 1468 and 1472 (Fig. 3A). The location of the 3′ end of the *nor-1* transcript observed in the cDNA nucleotide sequence agrees with the location of the transcription polyadenylation site mapped by RNase protection. The actual size of the transcript observed in Northern analysis also agrees with the size predicted in these transcript mapping analyses. The *nor-1* transcript was estimated to be 1.25 kb in length.

Disruption of *nor-1* and analysis of disrupted strains. To confirm the function of *nor-1* in the aflatoxin biosynthetic pathway, the plasmid pNA51-82 was created for the disruption of *nor-1* by one-step gene replacement (Fig. 1). Double recombination between *nor-1* coding or flanking regions on pNA51-82 and *nor-1* coding or flanking regions on the chromosome should result in a nonfunctional *nor-1* gene in the chromosome. Linearized (*Sph*I) or circular pNA51-82 was used in separate experiments to transform *A. parasiticus* NR-3 (*niaD*). The transformation frequency with the linearized vector was approximately 4 to 10 nitrate-utilizing transfor-

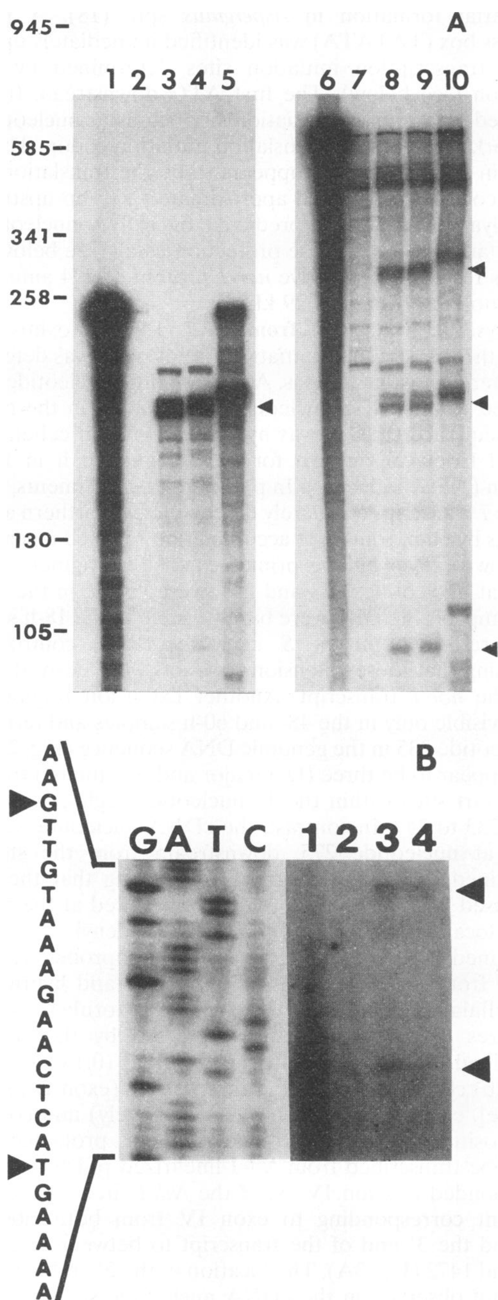

FIG. 3. Primer extension analysis and RNase protection assay of the 5′ and 3′ ends of *nor-1*. (A) RNase protection of the 3′ end of *nor-1*. A 0.251-kb riboprobe, transcribed from *Nde*I-linearized pSK07 (lanes 1 to 5), and a 0.758-kb riboprobe, transcribed from *Pst*I-linearized pSK07, were hybridized to tRNA from *S. cerevisiae* (lanes 1, 2, 6, and 7) or total cellular RNA of *A. parasiticus* (lanes 3 to 5 and 8 to 10). The hybridization mix was subsequently treated with a mixture of RNase A (2.5 U/ml) and RNase T1 (100 U/ml) (lanes 3 and 8) or with RNase T1 only (50 U/ml) (lanes 5 and 10). Protected fragments (186 bp [lanes 3 to 5] and 288, 182, and 100 bp [lanes 8 to 10]) are indicated by arrowheads. DNA markers from a *Sau*3A digest of pUC9 labelled with ^{32}P are indicated. Differences in migration of DNA and RNA may be as great as 5%. (B) Primer extension analysis of the 5′ end of the *nor-1* transcript. A 20-bp primer was radiolabelled and hybridized to torula yeast tRNA (lane 1) or total RNA from 18-h (lane 2), 48-h (lane 3), and 60-h (lane 4) induced cultures from *A. parasiticus*. Two extension fragments are indicated by arrowheads. A section of nucleotide sequence of pSK05 complementary to bases 233 to 253 (Fig. 2), with the same oligonucleotide primer and comigrating with the extension fragments, is shown on the left as a size reference.

mants per 100 regenerable protoplasts. In three separate experiments, between 10 and 54% of the nitrate-utilizing transformants accumulated an orange pigment on CAM which later was shown to be NA by TLC and UV-visible light spectrum analyses. The frequency of transformation to nitrate utilization with the circular plasmid pNA51-82 was similar to that with the linearized plasmid, but the percentage of NA-accumulating strains was less than 2% of the nitrate-utilizing transformants. No transformants were obtained when DNA was not present in the transformation mixture, and no NA-accumulating transformants were observed when only pSL82, carrying the *niaD*-selectable marker, was used as a control plasmid.

Southern hybridization analysis was performed on genomic DNA isolated from the parental strain NR-3 (*niaD*), from *niaD*$^+$ NA-accumulating transformants, and from *niaD*$^+$ aflatoxin-accumulating transformants (Fig. 4). Genomic DNA was cut with *Hin*dIII, blotted onto nylon membranes, and probed with a radiolabelled (with ^{32}P) riboprobe transcribed from pSK07. A 3.2-kb DNA fragment hybridized to the riboprobe in the DNA from strain NR-3 and in AFB1-accumulating transformants. As expected, this fragment was replaced with a 4.2-kb *Hin*dIII fragment in the *niaD*$^+$, NA-accumulating strains (Fig. 1). No other DNA fragment hybridized with the probe in these transformants, indicating that *nor-1* was disrupted by homologous recombination resulting in the replacement of the wild-type gene. The same blot was stripped to remove the riboprobe and reprobed with radiolabelled pUC19 (data not shown). Vector sequences were observed in the *niaD*$^+$, NA-accumulating transformants (disruptants) generated with the circular vector but not in the *niaD*$^+$, NA-accumulating strains, the *niaD*$^+$, aflatoxin-accumulating strains generated with the linear construct, nor in NR-3. The same genomic DNA was cut with *Bam*HI and reprobed with pSK07 and pUC19 (data not shown). These data were consistent with results observed for *Hin*dIII digests. The absence of vector sequences in the *niaD*$^+$, aflatoxin-accumulating strains indicated that these transformants were formed by gene replacement recombination at the *niaD* locus.

Five transformants that accumulated an orange pigment on CAM and aflatoxin-producing strain NR-3 were grown in YES medium (aflatoxin inducing) for further analysis. TLC analysis revealed a loss of AFB1 and AFG1 production in the transformants when compared with NR-3. TLC analyses of two representative transformants and NR-3 are shown in Fig. 5. The orange pigment, which comigrated with an NA standard, accumulated primarily in the mycelia of the transformants and was not observed in cultures of NR-3. Aflatoxins are known to pass efficiently through the mycelial walls to the surrounding growth medium (24), whereas some intermediates of aflatoxin biosynthesis, such as NA, have been shown to accumulate primarily in the mycelium (15). The absorbance spectrum of the orange pigment was indistinguishable from the spectrum for NA published previously (13) and from the spectrum for the NA standard used in this experiment. Direct competitive ELISA analysis to quantify amounts of AFB1 in the culture fluid revealed an approximately 20-fold greater accumulation of AFB1 in cultures of NR-3 (6.4 ± 0.3 mg of AFB1 per g [dry weight] of mycelium [mean ± standard deviation]) when compared with that of the transformed strains (0.327 mg ±

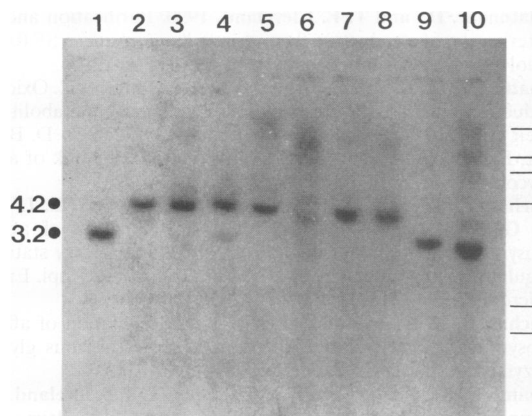

FIG. 4. Southern hybridization analysis of genomic DNA from the disrupted transformants. DNA was cut with *Hin*dIII and separated on an 0.8% agarose gel. Lanes: 1, *A. parasiticus* NR-3; 2 to 4, transformants disrupted with circular pNA51-82; 5 to 8, transformants disrupted with linearized (*Sph*I) pNA51-82; 9 and 10, aflatoxin-producing, nitrate-utilizing transformants. A radiolabelled riboprobe transcribed from pSK07 was used as a probe. The DNA size markers indicated on right are a *Hin*dIII digest of bacteriophage lambda (from the top, 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb). Film was exposed for 5 days at −81°C.

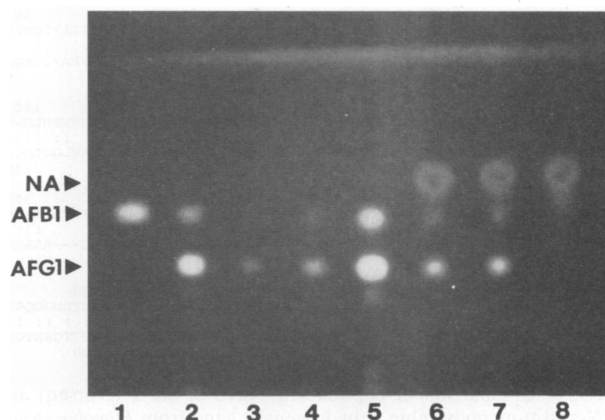

FIG. 5. TLC analysis of pigment extracts from aflatoxin-induced cultures of NR-3 (lanes 2 and 5) and two *nor-1*-disrupted transformants (lanes 3, 4, 6, and 7). Extracts from growth medium (lanes 2 to 4) and mycelial mats (lanes 5 to 7) are shown. AFB1 (lane 1) and NA (lane 8) were used as standards. TLC plates were observed under long-wave UV light.

0.16 mg of AFB1 per g [dry weight] of mycelium). These data strongly suggest that disruption of the *nor-1* gene results directly in accumulation of NA and a corresponding substantial decrease in AFB1 accumulation.

DISCUSSION

Recombinational inactivation provides the first direct evidence that *nor-1* is functionally involved in aflatoxin production. Disruption of a suppressor gene or a gene encoding an enzyme that fortuitously acted on the NA substrate would not give these results. In agreement with a previous study (42) involving gene disruption in *A. parasiticus*, linearization of the vector greatly increased the percentage of successful disruptants, whereas the circular vector also resulted in disruption of *nor-1* but at a much lower percentage. Cells transformed with circular pNA51-82 retained the plasmid vector, indicating that a multiple-step gene disruption may have occurred. In support of this hypothesis, NA-accumulating sectors appeared in aflatoxin-producing colonies 3 to 7 days after they had been transferred to CAM, suggesting that one recombinational event resulted in conversion to *niaD*$^+$, whereas a second resulted in disruption of *nor-1*. In contrast, the disrupted transformants produced by linearized pNA51-82 accumulated NA as soon as they were transferred to CAM. Further genetic analyses of these clones is required to define the exact mechanism of disruption with circular pNA51-82.

Whereas the direct role of *nor-1* in AFB1 biosynthesis is clear, the activity of the *nor-1* protein remains undescribed. To begin to understand the possible activity of this protein, the proposed amino acid sequence of *nor-1* was used to search the EMBL and GenBank database libraries with the Wisconsin Genetics Computer Group TFastA and Motif Search protocols. Three separate observations in these analyses provide evidence that *nor-1* encodes a ketoreductase, an activity that is consistent with the proposed role of the *nor-1* gene product in reduction of a keto group to a hydroxyl group during conversion of NA to AVN. (i) A motif search suggested that the *nor-1* protein belongs to the family of short-chain alcohol dehydrogenases (30, 40). The amino acid motif Tyr-Gly-Val-Ser-Lys-Leu-Ala-Ala-Asn-Tyr-Met found in short-chain alcohol dehydrogenases was observed in the *nor-1* protein sequence beginning at amino acid 291 (Fig. 2). The enzymes in this family are dehydrogenases/reductases that catalyze the reversible oxidation of hydroxyl groups to keto groups with the concomitant reduction of NAD(P). (ii) The second exon of the predicted *nor-1* protein contains a sequence similar to the conserved adenine nucleotide binding motif Gly-X-Gly-X-X-Ala found in nucleotide-binding proteins (19, 38), including the *ver-1* protein (35), although in the *nor-1* protein the sequence is Gly-X-Gly-X-X-Leu, where leucine is a conservative substitution of alanine. Conversion of NA to AVN theoretically requires concomitant oxidation of NADPH. A sequence (Tyr-Leu-Val-Thr) just upstream from the adenine nucleotide binding motif was also identified near the N terminus of several other proteins associated with secondary metabolites. These proteins include gene products of *ver-1* (Ala-Leu-Val-Thr) (35), *omt-1* (Tyr-Leu-Val-Thr) (45), *N*-acyl-D-mannosamine dehydrogenase from *Flavobacterium* sp. (Ala-Ile-Val-Thr) (44), and both of the proposed oxidoreductases (ORF 5 and 6) of the polyketide synthase from *Streptomyces violacearum* associated with the production of granaticin (Ala-Leu-Val-Thr) (34). The function of this sequence is not known at this time. (iii) Comparison of the proposed amino acid sequence of *nor-1* with the amino acid sequence of *N*-acyl-D-mannosamine dehydrogenase from *Flavobacterium* sp. (Fig. 6) revealed 23.2% identity and 50% similarity over a 211-amino-acid region. Comparison of the amino acid sequence of *nor-1* with the amino acid sequence of *ver-1* from *A. parasiticus* and the *Streptomyces violaceoruber* polyketide synthase (ORF 5) associated with production of the anthraquinone granatacin revealed identities of 26 and 32% and similarities of 75% in 120- and 103-amino-acid overlaps, respectively. The identifying short-chain alcohol dehydrogenase motif was also present in each of these protein sequences. Because *nor-1* shares a high degree of similarity with other NAD(P)-binding dehydrogenases/reductases over extended regions of the proteins, the data strongly suggest that it functions as a nucleotide-binding dehydrogenase/reductase.

The enzymatic activity encoded by *nor-1* is being investigated

```
                       10        20        30        40        50
Norseq           MNGSLSQHDQERLSTPYRDGPPEETVYLVTGASRGIGRGLIEAFLQRPKS
                                  | |: :::: :||||: ||||: :||:|:   |
Fvbnam GQAQRAIDGRTLPGGRVTSMTTAGVSRRPGRLAGKAAIVTGAAGGIGRATVEAYLREGAS
               10        20        30        40        50        60

             60        70        80        90        100       110
Norseq TVVACVRNVATATPALSALTVAEGSRMIIVQLNCDSETDAQAAVQTLREEHGVTHLDVVV
       :|      : : :| |:|   :| :    ::::|| :: |: :::   :   :: ||::|
Fvbnam VV------AMDLAPRLAATRYEEPG---AIPIACDLADRAAIDAAMADAVARLGGLDILV
                     70           80        90       100       110

            120       130       140        150       160       170
Norseq ANAAMATNFGPASTMPLEHLQAHMMVNMYAPVLLFQA-TRLMLQQSKQQAKFVLIGAPIS
       |::|: :: |   ::: :: : :: ||| :: |  :| :| |::::  ::     :|:|
Fvbnam AGGALKGGTGNFLDLSDADWDRYVDVNMTGTFLTCRAGARAMVAAGAGKDG---RSARII
             120       130       140       150       160

             180        190       200       210         220
Norseq TITNMHDYARAP-LTAYGVSKLAANYMVRKFHFENKWLTAFI--IDPGHVQTDMGDQGAR
       ||::::::  :|  :|| ::| ::: ::| :  :  : : ::  |:||:|::: :: |
Fvbnam TIGSVNSFMAEPEAAAYVAAKGGVAMLTRAMAVDLARHGILVNMIAPGPVDVTGNNTGYS
      170       180       190       200       210       220
```

FIG. 6. Comparison of peptide sequences of *nor-1* (Norseq) and the *N*-acyl-D-mannosamine dehydrogenase gene from *Flavobacterium* sp. strain 141-8 (Fvbnam). Identical amino acids (dashed lines) and conservative substitutions (dotted lines) are indicated. The small-chain alcohol dehydrogenase motif is underlined. The proposed adenine nucleotide-binding domain is indicated in bold.

further through the production of a fusion protein for expression in *E. coli*. Assays of the activity of the fusion protein product should result in clarification of the activity encoded by *nor-1*. The size of the predicted *nor-1* protein, 29 kDa, differs from that of the NA reductase purified by Bhatnagar and Cleveland (40 kDa) (4). The size of another NA reductase, purified by Chuturgoon et al. (11), has not been reported. However, the continued presence of aflatoxin in the cultures of the strains disrupted at *nor-1* supports the hypothesis that an alternative pathway(s) for conversion of NA to AVN exists or that there are two or more enzymes present with similar activities. Southern analysis of the genomic DNA of *A. parasiticus* presented here reveals the presence of a single *nor-1* gene, but this does not preclude the possibility of a nonhomologous gene with the same activity.

Isolation and identification of structural and regulatory genes from the aflatoxin pathway provide a unique opportunity to study the regulation of production of these secondary metabolites in *Aspergillus* spp. Reporter gene fusions of the *nor-1* and *ver-1* promoter regions to the gene for β-glucuronidase have been constructed for the identification of *cis*-acting regions responsible for regulating the aflatoxin biosynthetic pathway. Generation of a promoter deletion series from these fusions and gel shift assays of these regions could reveal the areas important to regulation of these genes. An understanding of the regulation of this pathway could lead to novel approaches to the elimination of aflatoxin from food supplies worldwide.

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