Chromosomal Location Plays a Role in Regulation of Aflatoxin Gene Expression in *Aspergillus parasiticus*

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The *nor-1* gene in the filamentous fungus *Aspergillus parasiticus* encodes a ketoreductase involved in aflatoxin biosynthesis. To study environmental influences on *nor-1* expression, we generated plasmid pAPGUSNNB containing a *nor-1* promoter–β-glucuronidase (GUS) (encoded by *uidA*) reporter fusion with *niaD* (encodes nitrate reductase) as a selectable marker. *niaD* transformants of *A. parasiticus* strain NR-1 (*niaD*) carried pAPGUSNNB integrated predominantly at the *nor-1* or *niaD* locus. Expression of the native *nor-1* and *nor-1*::GUS reporter was compared in transformants grown under aflatoxin-inducing conditions by Northern and Western analyses and by qualitative and quantitative GUS activity assays. The timing and level of *nor-1* promoter function with pAPGUSNNB integrated at *nor-1* was similar to that observed for the native *nor-1* gene. In contrast, *nor-1* promoter activity in pAPGUSNNB and a second *nor-1*::GUS reporter construct, pBNG3.0, was not detectable when integration occurred at *niaD*. Because *niaD*-dependent regulation could account for the absence of expression at *niaD*, a third chromosomal location was analyzed using pAPGUSNP, which contained *nor-1*::GUS plus *pyrG* (encodes OMP decarboxylase) as a selectable marker. GUS expression was detectable only when pAPGUSNP integrated at *nor-1* and was not detectable at *pyrG*, even under growth conditions that required *pyrG* expression. *nor-1*::GUS is regulated similarly to the native *nor-1* gene when it is integrated at its homologous site within the aflatoxin gene cluster but is not expressed at native *nor-1* levels at two locations outside of the aflatoxin gene cluster. We conclude that the GUS reporter system can be used effectively to measure *nor-1* promoter activity and that *nor-1* is subject to position-dependent regulation in the *A. parasiticus* chromosome.

Aflatoxins are highly toxic secondary metabolites produced predominantly by the imperfect fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (4–6, 18, 23). These toxins frequently contaminate food and feed crops, including peanuts, corn, cottonseed, and tree nuts, and generate a large health and economic impact in the United States and in other countries (4). To help eliminate aflatoxin contamination of food and feed, we have studied the mechanisms that regulate aflatoxin gene expression. This knowledge will be used to design novel, effective control strategies to reduce aflatoxin contamination of crops in the field and during storage.

Aflatoxin synthesis requires at least 16 enzyme activities (16) encoded by up to 25 individual genes (10). These genes are clustered in *A. flavus*, *A. parasiticus*, and *A. nidulans* (*A. nidulans* synthesizes the late aflatoxin pathway intermediate sterigmatocystin) (4, 10, 16, 23, 24, 27). It was previously hypothesized that clustering of aflatoxin genes may allow coregulation in response to environmental cues, although no conclusive data were reported (23).

Preliminary evidence for a role for clustering in aflatoxin gene regulation was reported by Liang et al. (14). The *ver-1* promoter, which drives expression of one aflatoxin structural gene, was fused to *uidA* (encodes β-glucuronidase [GUS]) (9) to generate a reporter plasmid, pHD6.6, containing *niaD* (encodes nitrate reductase) as a selectable marker (8). pHD6.6 was transformed into *A. parasiticus* NR-1 (*niaD*), resulting in integration predominantly at the *ver-1* or *niaD* locus via homologous recombination. Single-copy integration of pHD6.6 at *niaD* resulted in a 500-fold reduction in *ver-1* promoter activity when compared with single-copy integration at the *ver-1* locus; however, the temporal pattern of expression appeared to be similar at both loci. One explanation for reduced *ver-1* promoter function at the *niaD* locus is that the location in the aflatoxin cluster results in positive, position-dependent regulation of *ver-1* expression. An alternative hypothesis is that expression of *ver-1* integrated at *niaD* is negatively influenced by *niaD* regulation. In *A. nidulans*, *niaD* expression is under dual control: (i) nitrogen catabolite repression is mediated by *areA*, and (ii) pathway specific induction is mediated by *nirA* (17). In the absence of ammonia or glutamine and in the presence of nitrate, *niaD* is expressed. Under the rich growth conditions utilized by Liang et al. (14), *niaD* may have been repressed, and therefore the lack of *ver-1* expression at the *niaD* site could have resulted from *niaD*-dependent regulation.

Our goals in this study were (i) to generate a *nor-1*::GUS reporter construct and validate its use for monitoring effects of growth environment on *nor-1* promoter function and (ii) to monitor *nor-1* promoter activity when integrated at different chromosomal loci. The data demonstrate the utility of the GUS reporter as a sensitive and reproducible means to mon-

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itor environmental influences on *nor-1* expression. The cloned *nor-1* promoter functioned similarly to the native *nor-1* promoter at its appropriate location within the aflatoxin gene cluster, but not outside this cluster, providing us with clear supporting evidence for the hypothesis that the aflatoxin gene cluster exerts a positive position-dependent regulatory influence on the *nor-1* promoter.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* DH5α F′ᵉ [F′ *endA1 hsdR17* (r$_k^-$ m$_k^-$) *supE44 thi-1 recA1 gyrA* (Nal^r) Δ*relA1* (*lacZYA argF*)$_{u169}$:(m80 Δ*lacZ M15*)] (Gibco BRL, Life Technologies Inc., Rockville, Md.) was used to amplify plasmid DNA using standard procedures (1). *A. parasiticus* NRRL 5862 (SU1; ATCC 56775) was used as the wild-type aflatoxin-producing strain. *A. parasiticus* NR-1 (*niaD*) was derived from *A. parasiticus* SU1 by spontaneous mutation (8) and served as the recipient strain for reporter plasmids containing the *niaD* selectable marker (pAPGUSNNB; pBNG3.0). *A. parasiticus* CS10 (*wh-1 ver-1 pyrG*) (20) was derived from *A. parasiticus* ATCC 36537 (*wh-1 ver-1*) by nitrosoguanidine mutagenesis and was used as a recipient for the reporter construct carrying the *pyrG* selectable marker (pAPGUSNP).

To measure *nor-1* promoter activity via GUS activity assay, to analyze dry weight, to quantify aflatoxin accumulation, and to isolate RNA and protein, fungi were grown in liquid shake culture (batch fermentation) at 29°C in the dark, with shaking at 150 rpm as previously described (14). The culture media consisted of YES liquid (2% yeast extract, 6% sucrose; pH 5.8) and solid medium (1.5% agar). To grow the recipient strain CS10, YES medium or Czapek-Dox medium (CZ; Difco, Detroit, Mich.) was supplemented with uridine (100 µg/ml).

Plasmid constructs. (i) pAPGUSNNB. The pAPGUSNNB *nor-1*::GUS fusion construct was derived from pAPGUSN (Fig. 1A) by insertion of a 7.4-kb *Sal*I/*Xho*I fragment carrying *niaD* from *A. parasiticus* (8). This restriction fragment was isolated from plasmid pSL82 (8). The *nor-1* promoter region (5′ *nor-1*) consists of a 3-kb *Hin*dIII DNA restriction fragment carrying flanking sequences, promoter, and the first 21 amino acid residues of Nor-1 fused in frame to *uidA* from *E. coli* (9). The *nor-1* terminator region (3′ *nor-1*) is a 1.8-kb *Eco*RI/*Sal*I genomic DNA fragment containing the last six amino acids of Nor-1, the transcription terminator, and flanking sequences. Note that *niaD* is transcribed from the opposite DNA strand as *nor-1*::GUS (divergent promoters).

(ii) pAPGUSNP. pAPGUSN was digested with *Nhe*I and the ends were filled in with the Klenow fragment of DNA polymerase I (Roche Molecular Biochemicals, Indianapolis, Ind.). The 2.5-kb *pyrG* gene was amplified by PCR with *Taq* DNA polymerase (Gibco BRL), pPG3J (20) as template, and appropriate primers (5′-GTAGAAGTTGTTCAGTAGCTGATGG-3′ and 5′-CGAGTATCACAGTCAGGACTCCACG-3′). The resulting PCR fragment was end polished by *Pfu* polymerase (Stratagene, LaJolla, Calif.) and blunt-end ligated into pAPGUSN to generate pAPGUSNP.

(iii) pBNG3.0. pNEB193 (New England Biolabs, Beverly, Mass.) was first cut with *Bam*HI, the protruding ends were filled in by Klenow fragment, and a *Not*I linker (5′-AGCGGCCGCT-3′) was ligated onto the blunt ends, creating pNebN1. Next, the 7.4-kb *Xho*I/*Sal*I fragment from pSL82 (8) containing the *niaD* gene was ligated into the *Sal*I site in pNebN1 to create pNiaD-A1. To insert the *uidA* gene (GUS), the reporter construct pAPGUSNNB was utilized as template in a PCR. Primers were designed to amplify a 4-kb fragment from pAPGUSNNB that contained the terminator region, the GUS coding sequence, and the *nor-1* coding sequence upstream of the fusion point. The primers carried restriction enzyme sites to facilitate cloning: the 5′ primer (5′-TAGCGGCCGCGATCAAGAGAAGCTCTATACG-3′) contained *Not*I and the 3′ primer (5′-TTGGCGCGCCCTCGATGATGATGCTCTG-3′) contained *Asc*I. Since the *Not*I site was located within the coding sequence, care was taken to maintain the correct reading frame. The 4-kb *Not*I/*Asc*I PCR product was cloned into the *Not*I and *Asc*I sites in pNiaD-A1, resulting in pNANG-3F. Finally, a 3.0-kb *nor-1* promoter piece was amplified by using PCR with pAPGUSNNB as template and appropriate primers with *Not*I tails (5′-TCGCGGCCGCTAAGTGATCCATTCATTATGTC-3′ and 5′-TTGCGGCCGCTCCTTGTCTCTGTACTGATAAA-3′). In this way, the *nor-1* promoter could be easily removed and replaced with other putative promoters to test for function. The nucleotide sequence from pBNG3.0 was analyzed from the *nor-1*::GUS fusion site to 332 bp upstream from the transcriptional start site and confirmed the correct nucleotide sequence in this reporter construct.

(iv) pGAPN2B. The pGAPN2B plasmid was used as a control for constitutive GUS expression. It contains the β-tubulin promoter from *A. flavus* fused in frame to *uidA* (β-tubulin::GUS) and *A. parasiticus niaD* as a selectable marker.

Transformation and identification of the site of plasmid integration. (i) Transformation. For transformation of NR-1, protoplasts were generated and transformed essentially as described previously (8). Selection of transformants was conducted on CZ agar medium with 20% sucrose as an osmotic stabilizer. Agar plates were incubated in the dark at 29°C for 3 to 5 days. The same procedure was used to generate protoplasts for transformation of *A. parasiticus* CS10 except mycelia were grown in CZ medium supplemented with uridine (100 µg/ml).

(ii) Rapid DNA extraction procedure for PCR analysis. Transformants were grown until sporulation (usually 3 to 5 days) and conidiospores were transferred to duplicate CZ agar plates using a sterile toothpick or sterile pipette tip. After 3 to 4 days (or as soon as colonies could be observed), colonies were analyzed. A sterile 200-µl plastic pipette tip was used to transfer a portion of the fungal colony (including conidiospores and mycelia) into a microcentrifuge tube containing 100 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The process was repeated up to three times to ensure that sufficient fungal cells were transferred. Tubes were heated in a boiling water bath for 5 min and placed on ice. The samples were centrifuged at 12,000 × g for 5 min at 4°C. The supernatant (fungal extract) containing DNA was transferred to a new microcentrifuge tube. The extract was diluted with TE buffer (undiluted, 1:5, 1:20, and 1:100). In most assays, 2.5 µl of the 1:5 dilution resulted in good amplification in a 50-µl PCR. The remainder of the fungal extract was stored at −80°C and could be analyzed by PCR for at least 3 months. Diluted samples stored at 4°C could be analyzed for at least 1 week.

(iii) PCR analysis. PCR analysis was conducted on DNA extracted from *A. parasiticus* transformants and the recipient strains *A. parasiticus* CS10 and NR-1. The primer pairs utilized depended upon the plasmid vector used and the site of integration analyzed (Table 1). A 50-µl reaction consisted of 2.5 µl of the diluted fungal extract, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.15% Triton X-100, 0.1 mg of bovine serum albumin per ml, 0.5 to 1.0 µM (each) primer, 200 µM (each) dNTPs, 2.0 to 3.0 mM MgCl₂, and either 2.5 U of *Pfu* Turbo polymerase (2.5 U/µl; Stratagene) or 2.5 U of *Taq* polymerase (5 U/µl) (Gibco BRL). PCR cycling depended on primer design and the target size (Table 1). PCR was performed in a Gene Ampli System 9600 (Perkin-Elmer) thermal cycler. All PCRs were initiated by a denaturation step at 95°C for 3 min followed by 35 cycles of denaturation, annealing, and extension. Reactions were terminated with an extension reaction at 72°C for 10 min. PCR products were observed by electrophoresis on a 1% agarose gel using standard procedures (1).

Measurement of mycelial dry weight and aflatoxin. Mycelial dry weight and aflatoxin were measured by enzyme-linked immunosorbent assay as described previously (19).

GUS activity assays. (i) Qualitative assay. Transformants were transferred from selective medium to agar plates containing an aflatoxin-inducing solid growth medium (YES) overlaid with sterilized circular 82-mm-diameter Nytran Plus membranes (Schleicher and Schuell Inc., Keene, N.H.). Plates were incubated in the dark at 29°C for 36 to 55 h. Membranes with attached fungal colonies were removed from the surface of the agar plates and immersed in liquid nitrogen for 30 s (13). Membranes were allowed to thaw and immersed again in liquid nitrogen for 30 s. After thawing, membranes were incubated in GUS substrate solution (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 0.27% β-mercaptoethanol, 0.04% 5-bromo-4-chloro-3-indolyl-glucuronide [Gold Biotechnology, St. Louis, Mo.]) for up to 24 h. Positive GUS activity resulted in a blue color in the colony.

(ii) Quantitative assay. Samples of mycelia (either stored frozen at −80°C after harvest or frozen in liquid nitrogen immediately before the assay) were ground with a mortar and pestle in liquid nitrogen. Approximately 100 mg of crushed tissue was mixed with 500 µl of GUS lysis buffer (50 mM NaH₂PO₄ [pH 7.0], 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sulfate, 10 mM β-mercaptoethanol, 25 µg of phenylmethylsulfonyl fluoride per ml) and centrifuged at 12,000 × g at 4°C for 10 min. The protein concentration was determined by the Bradford method (2) using bovine serum albumin as a protein standard and Protein Assay Dye reagent (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions. One microgram of total protein from each extract was incubated in a 200-µl volume of 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) at 37°C for up to 30 min as necessary. To determine GUS activity, the fluorescence of each reaction mixture was measured at an excitation wavelength of 365 nm and an emission wavelength of 455 nm with an SLM-4000 fluorometer (SLM Instruments, Champaign, Ill.). GUS activity was expressed as nanomoles of 4-methylumbelliferone generated per minute per milligram of protein.
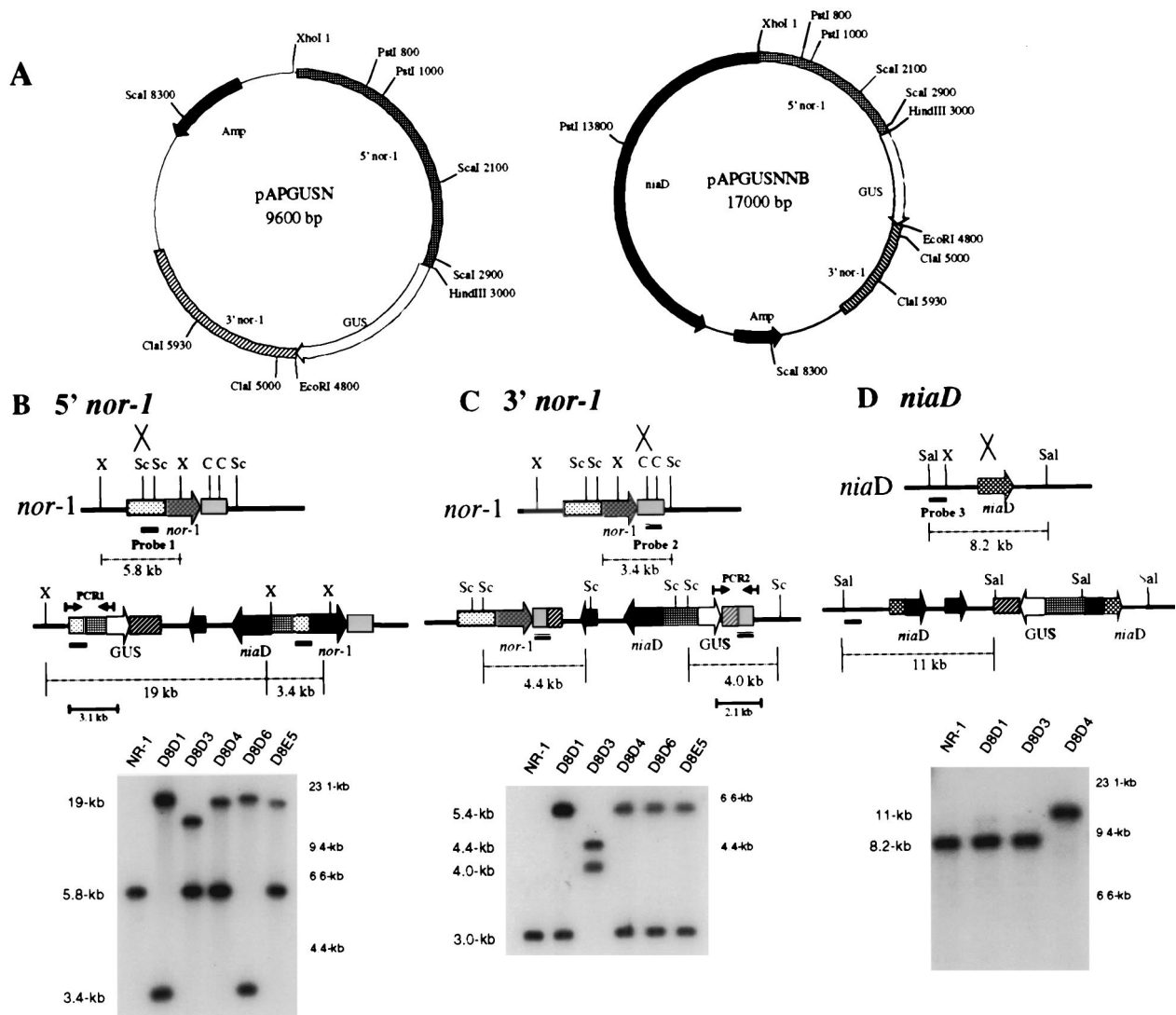
FIG. 1. Southern hybridization analysis of site of integration in pAPGUSNNB transformants. (A) Restriction endonuclease maps of pAPG USN and pAPGUSNNB. Only restriction endonuclease sites relevant to this study are shown. (B) Schematic for Southern hybridization and PCR analyses of pAPGUSNNB integration into the 5′ region of *nor-1* and Southern hybridization data. *A. parasiticus* genomic DNA was digested with *Xho*I and probed with probe 1. Single-crossover integration of pAPGUSNNB into the 5′ region of *nor-1* results in two bands, of 19 and 3.4 kb, and the disappearance of the 5.8-kb wild-type band. (C) Schematic for Southern hybridization and PCR analysis of pAPGUSNNB integration into the 3′ region of *nor-1* and Southern hybridization data. Genomic DNA was digested with *Sca*I and probed with probe 2. Single-crossover integration of pAPGUSNNB into the 3′ region of *nor-1* results in two bands, of 4.0 and 4.4 kb, and the disappearance of the 3.0-kb wild-type band. (D) Schematic for Southern hybridization and PCR analysis of pAPGUSNNB integration into *niaD* and Southern hybridization data. Genomic DNA was digested with *Sal*I and probed with probe 3. Single-crossover integration of pAPGUSNNB into *niaD* results in an 11-kb Southern hybridization signal and the disappearance of the 8.2-kb wild-type band. Abbreviations: X, *Xho*I; Sc, *Sca*I; C, *Cla*I; Sal, *Sal*I. The numbers in kilobases to the right of the Southern blots in panels B, C, and D represent molecular size markers. The numbers in kilobases to the left represent the sizes of actual restriction fragments observed in the analysis.

Southern and Northern hybridization analyses. DNA and RNA were isolated and hybridization analyses were conducted essentially as described previously (19) using standard procedures (1). Restriction enzyme digestion and probes used to confirm the site of integration (see Fig. 1, 5, and 8) were as follows.

(i) pAPGUSNNB, 5′ nor-1 integration. Genomic DNA of transformants was digested with *Xho*I and probed with a 750-bp *Sca*I fragment (probe 1) isolated from the 5′ region of *nor-1* in pAPGUSNNB. DNA from *A. parasiticus* NR-1 generates a 5.8-kb band, while DNA from a 5′ integrant of pAPGUSNNB generates 19- and 3.4-kb bands.

(ii) pAPGUSNNB, 3′ nor-1 integration. Genomic DNA of transformants was digested with *Sca*I and probed with a 900-bp *Cla*I fragment isolated from the 3′ region of *nor-1* in pAPGUSNNB (probe 2). DNA from the recipient strain *A. parasiticus* NR-1 generates a 3.0-kb band, while DNA from a 3′ integrant of pAPGUSNNB generates 4.0- and 4.4-kb bands.

(iii) pAPGUSNNB, niaD integration. Genomic DNA of transformants was digested with *Sal*I and probed with an 800-bp *Sal*I/*Xho*I fragment (probe 3) isolated from *niaD* in pSL82 (8). DNA from *A. parasiticus* NR-1 generates an 8.2-kb band, while DNA from a *niaD* integrant of pAPGUSNNB generates an 11-kb band.

(iv) pBNG3.0, 3′ nor-1 integration. Genomic DNA of transformants was digested with *Sca*I and probed with probe 2. DNA from *A. parasiticus* NR-1 generates a 3.0-kb band, while DNA from a 3′ integrant of pBNG3.0 generates 3.7- and 4.0-kb bands.

TABLE 1. Primers and PCR parameters for analysis of DNA templates prepared by the boiling method

Primer[a]	Target size (kb)	Primer sequence (5′-3′)	PCR cycling parameters
PCR1			
JL99	3.1	TTT CAC GGG TTG GGG TTT CTA CAG G	95°C for 1 min, 62°C for 1 min, 72°C for 5 min
JL100	3.1	GAC GGG GAA CCT CTT TAC AAA CAT C	
PCR2			
JL102	2.1	CGC AAG GTG AGG GTT CGA ACC GAG G	95°C for 1 min, 68°C for 1 min, 72°C for 3 min
JL103	2.1	CCG CAG CAG GGA GGC AAA CAA TGA A	
PCR3			
JL221	3.5	CCA GAA AAT GCG AAG GTA AGT GCT TC	95°C for 1 min, 55°C for 1 min, 72°C for 5 min
JL222	3.5	GCG ACA CGG AAA TGT TGA ATA CTC AT	
Control			
JL136	1.4	AGG CTC GAA AGG CGC ATA CGA	95°C for 1 min, 68°C for 1 min, 72°C for 2 min
JL137	1.4	GTG GTC TAC TGC CCA GCC ATC	

[a] PCR1 primers are for integration into the 5′ region of *nor-1* (pAPGUSNP, pBNG3.0), PCR2 primers are for integration into the 3′ region of *nor-1* (pAPGUSNP, pBNG3.0), PCR3 primers are for integration into *pyrG* (pAPGUSNP), and control primers are for endogenous *vbs* exon II (pAPGUSNP).

(v) pAPGUSNP, 5′ *nor-1* integration. Genomic DNA of transformants was digested with *Xho*I and probed with probe 1. DNA from *A. parasiticus* CS10 generates a 5.8-kb band, while DNA from a 5′ integrant of pAPGUSNP generates 13.0- and 3.4-kb bands.

(vi) pAPGUSNP, 3′ *nor-1* integration. Genomic DNA of transformants was digested with *Sca*I and probed with probe 2. DNA from *A. parasiticus* CS10 generates a 3.0-kb band, while DNA from a 3′ integrant of pAPGUSNP generates 4.0- and 4.4-kb bands.

(vii) pAPGUSNP, integration at *pyrG*. Genomic DNA of transformants was digested with *Sac*I and probed with a 2.5-kb *pyrG* fragment generated by PCR (probe 4). DNA from *A. parasiticus* CS10 generates an 8.1-kb band, while DNA from a *pyrG* integrant generates 5.9- and 14.3-kb bands.

Probes used for analysis of transcript accumulation via Northern analysis were as follows. For the GUS transcript, a 1.7-kb amplification product within the GUS coding region was generated using specific primers (5′-CACCGAAGTTC ATGCCAGTCCAGCG-3′ and 5′-CTCGACGGCCTGTGGGCATTCAGTC-3′). The GUS transcript is approximately 2.2 kb in size. For *nor-1* transcripts, a 780-bp PCR product was generated from a *nor-1* cDNA (29) using specific primers (5′-CGTCCCAAAAGCACC-3′ and 5′-GCGACACGAACCCAG-3′). The *nor-1* transcript was approximately 1.3 kb in size, in agreement with a value reported previously (19).

Probes for Southern and Northern analyses were radiolabeled with the Random Primed DNA labeling kit from Boehringer-Mannheim Biochemicals GmbH (Mannheim, Germany) using a protocol provided by the manufacturer. Alternatively, probes were prepared using digoxigenin and a nonradioactive detection kit and procedure (Roche Molecular Biochemicals).

Western blot analysis. Protein purification and Western blot analysis were conducted essentially as described previously (14). Rabbit polyclonal antibodies for GUS protein were purchased from Clontech Laboratories (Palo Alto, Calif.) and used according to instructions supplied by the manufacturer. Highly specific polyclonal antibodies for Nor-1 have been described previously (28; M. Miller, M. Rarick, R. Zhou, F. Trail, and J. E. Linz, submitted).

RESULTS

Transformation of *A. parasiticus* NR-1 with pAPGUSNNB and screening by qualitative GUS assay. Transformation of *A. parasiticus* strain NR-1 with plasmid pAPGUSNNB in three separate experiments resulted in transformation frequencies of 4.0×10^{-5}, 5.0×10^{-5}, and 5.1×10^{-5} CFU/protoplast using presumably saturating levels of DNA at 3 to 7 µg per transformation. *niaD*-positive colonies were screened for GUS activity in a qualitative assay (not shown). Thirty-five of 149 pAPGUSNNB transformants (23%) produced the GUS$^+$ phenotype.

Southern hybridization analysis of pAPGUSNNB transformants. Transforming DNA can integrate at either homologous or heterologous locations in the genomes of filamentous fungi. Southern hybridization analysis of pAPGUSNNB transformants identified three types of single-crossover recombinants as predicted (Fig. 1). Transformants D8D1 and D8D6 were GUS$^+$ and represented single-crossover integration of pAPG USNNB into the 5′ region of *nor-1* (Fig. 1B). Multiple integration of the vector may have occurred in D8D1, while D8D6 carried a single integrated copy of the vector. Transformant D8D3 was GUS$^+$ and resulted from a single-crossover integration into the 3′ region of *nor-1* (Fig. 1C). Transformant D8D4 was GUS$^-$ and resulted from single-crossover integration into the *niaD* locus (Fig. 1D). In a separate transformation experiment, 10 of 11 GUS$^+$ isolates had pAPGUSNNB integrated at either a 5′ *nor-1* locus (isolates 8, 34, 67, and 73) or a 3′ *nor-1* locus (isolates 50, 59, 61, 68, 85, and 96). The site of integration in the eleventh GUS$^+$ isolate (isolate 15) could not be conclusively assigned to either the 5′ or 3′ region of *nor-1*. However, the vector was clearly not integrated at *niaD*. In contrast, none of the six GUS$^-$ isolates had pAPGUSNNB at either a 5′ or 3′ *nor-1* locus. Two isolates (isolates 10 and 20) were *niaD* single-crossover integrants and four (isolates 25, 35, 40, and 45) were double-crossover gene replacements of the *niaD* selectable marker.

PCR analysis to determine integration site of pAPGUSNNB. As predicted (Fig. 1C), pAPGUSNNB transformants 50, 59, 61, 68, 85, and 96 produced a 2.1-kb amplicon with primers PCR2 (JL102 and JL103) (Fig. 2A). This amplicon is specific for pAPGUSNNB integration into the 3′ region of *nor-1*. Transformants 8, 34, 67, and 73 produced a 3.4-kb amplicon with primers PCR1 (JL99 and JL100), indicating 5′ *nor-1* integration of pAPGUSNNB (Fig. 2B). These PCR integration assays were confirmed by Southern hybridization analysis (data not shown). Transformants 10 and 15 did not produce PCR products in either *nor-1* integration PCR assay, supporting the Southern hybridization data showing transformant 10 to be a pAPGUSNNB *niaD* integrant and isolate 15 to be nondefinable (possible ectopic integration).

Comparison of *nor-1* and *uidA* transcription in pAPGU SNNB transformant D8D3. Transformant D8D3, with the *nor-1*::GUS reporter construct integrated into the 3′ region of
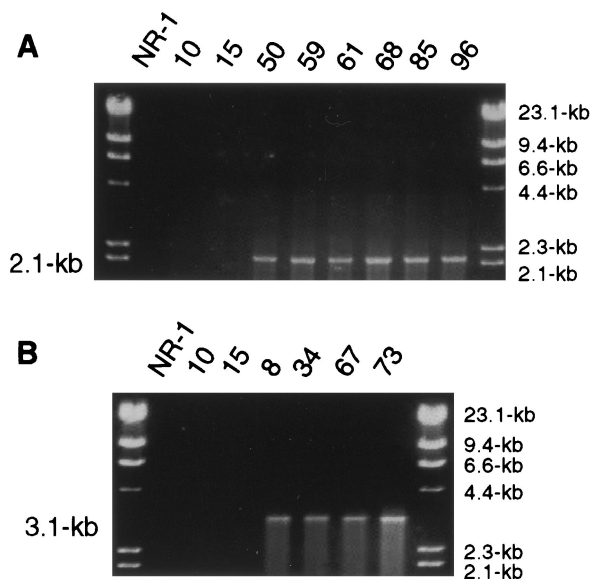
FIG. 2. PCR analysis of pAPGUSNNB transformants to detect 3′ and 5′ nor-1 integration. Genomic DNA was isolated from *A. parasiticus* and amplified by PCR using the primers (Table 1) represented in Fig. 1 by arrows (PCR 1 [5′ nor-1] and PCR2 [3′ nor-1]). (A) PCR analysis of 3′ nor-1 integrants of pAPGUSNNB (transformants 50, 59, 61, 68, 85, and 96). (B) PCR analysis of 5′ nor-1 integrants (transformants 8, 34, 67, and 73). NR-1 (parent strain) and transformants 10 (pAPGUSNNB integrated at *niaD*) and 15 (site of integration not completely defined) were used as negative controls.

nor-1, was grown in aflatoxin-inducing medium and the expression of nor-1 and uidA was analyzed 24, 48, and 72 h after inoculation. The dry weight of D8D3 (Fig. 3A) and control strains (NR-1, NR-1/pSL82, and NR-1/pGAPN2B) was similar (less than 15% variability) at each time point. Active growth occurred between 0 and 48 h (log phase). A transition from active growth to stationary phase was observed between 48 and 72 h as previously described (3, 12, 17, 20).

RNA isolated at 24, 48, and 72 h from D8D3 and each of the three control strains was subjected to Northern hybridization analysis using nor-1 and uidA PCR probes (Fig. 3B and C). The nor-1 transcript was not observed in D8D3 at 24 h but was detected at high levels at 48 and 72 h. uidA transcript accumulation in D8D3, though less abundant than nor-1, followed the same pattern of accumulation as the native nor-1 transcript, suggesting that both copies of the nor-1 promoter functioned similarly under these growth conditions. The specific activities of the nor-1 and uidA probes in these experiments were roughly equivalent.

The nor-1 transcript accumulation pattern was similar in each of the three control strains (Fig. 3B); however, the level of transcript in NR1/pSL82 and NR-1/pGAPN2B was lower than in the other strains. As expected, uidA transcript was not detected at any time point in NR-1 or NR-1/pSL82 but was present in NR-1/pGAPN2B at relatively high levels at 24 h and at decreasing concentrations at 48 and 72 h. This pattern of uidA transcript accumulation is significant because the promoter of the housekeeping gene (β-tubulin) is transcribed during active growth and then declines after the transition to stationary phase. This pattern differs from that for the aflatoxin

promoters, which are not actively transcribed until the transition from active growth to stationary phase.

Nor-1 and GUS protein accumulation patterns were similar to accumulation of their respective transcripts (data not shown), suggesting that nor-1 regulation under these growth conditions occurs primarily at the level of transcription.

Comparison of aflatoxin levels and GUS activity in pAPGU SNNB transformant D8D3. The same time course experiment that provided data on nor-1 and uidA transcription was also the source for data on aflatoxin levels and GUS activity. Neither aflatoxin nor GUS activity was detected in D8D3 at 24 h of fungal growth (Fig. 4), but relatively high levels of both were observed at 48 and 72 h. This pattern is consistent with the accumulation of nor-1 and uidA transcripts (Fig. 3B and C) from D8D3 mycelial samples. Although GUS activity in D8D3 increased slightly from 48 to 72 h, a greater increase in afla-
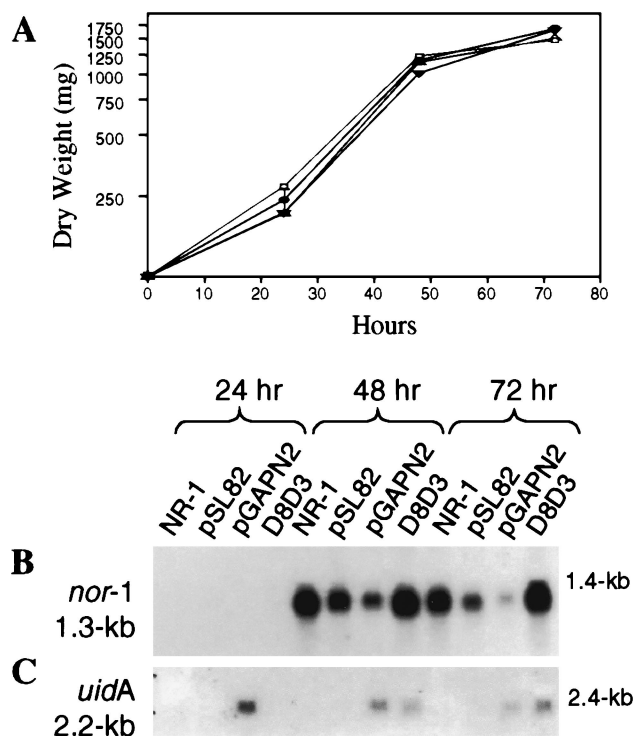

FIG. 3. Analysis of growth and expression of the nor-1 promoter in transformant D8D3 and control strains. (A) D8D3 (●) and control strains of *A. parasiticus*, NR-1 (△), NR-1/pSL82 (▽), and NR-1/pGAPN2B (□) were inoculated into an aflatoxin-inducing growth medium (YES broth). Mycelia were harvested at 24, 48, and 72 h after inoculation. A portion of each sample was dried for weight determination (milligrams per 100 ml of culture). For the control strains, the points on the graph represent data from a single culture. D8D3 was grown in triplicate and the error bars indicate standard errors. (B and C) Northern hybridization analysis of nor-1 and uidA transcripts. Thirty micrograms of total RNA was loaded into each lane. Each autoradiogram represents RNA isolated from mycelia after growth for 24, 48, and 72 h in aflatoxin-inducing medium. Blotted RNA was hybridized with a PCR-amplified 780-bp nor-1 probe (B) or a 1.7-kb amplification product of the uidA gene (C). The nor-1 transcript is approximately 1.3 kb and the predicted nor-1::GUS transcript is approximately 2.2 kb. This estimate is based on the size of the uidA gene in nor-1::GUS and the nor-1 transcription initiation and polyadenylation sites which are also included in the fusion.
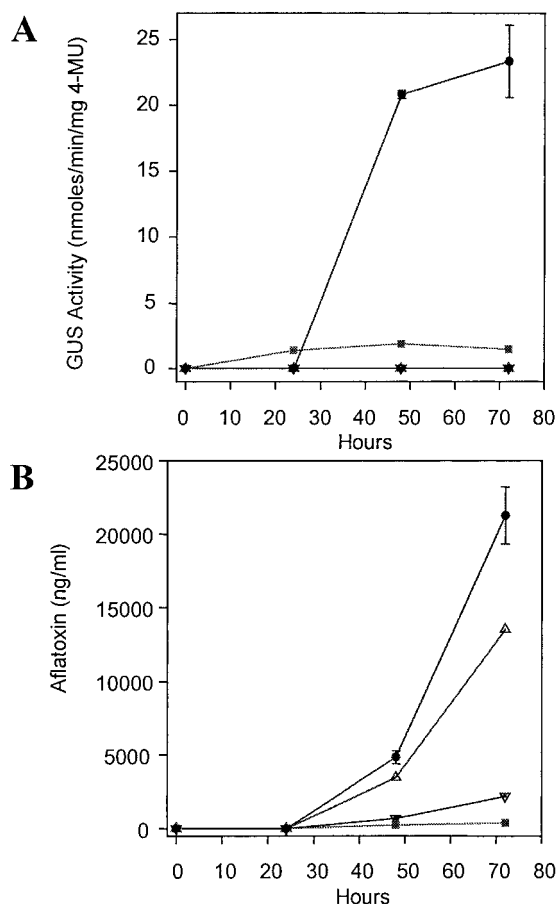
FIG. 4. GUS activity and aflatoxin production in transformant D8D3 and control strains. Mycelia of pAPGUSNNB transformant D8D3 (●) and control strains NR-1 (△), NR-1/pSL82 (▽), and NR-1/pGAPN2B (■) were harvested at 24, 48, and 72 h after inoculation in aflatoxin-inducing medium (YES broth). (A) One microgram of protein from each mycelial sample was used to determine GUS activity. The substrate for GUS analysis was MUG. 4MU, 4-methylumbelliferone. (B) Culture filtrate was used for the determination of aflatoxin levels via enzyme-linked immunosorbent assay. For the control strains, the points on the graph represent data from a single culture. D8D3 was grown in triplicate and the error bars indicate standard errors.

toxin levels during this period was observed. This difference in proportion could be explained by aflatoxin instability and would probably be absent if Nor-1 activity was measured directly.

The NR-1/pSL82 and NR-1/pGAPN2B controls were relatively weak producers of aflatoxin, as might be expected when comparing the *nor-1* transcripts of these strains with those of D8D3 and NR-1. GUS activity was absent in NR-1 and NR-1/pSL82 at all three time points and present in NR-1/pGAPN2B at all three time points. At 48 h, D8D3 expressed 11 times more GUS activity than NR-1/pGAPN2B, and at 72 h D8D3 expressed 48 times more activity. Since *uidA* transcript levels were similar in both of these transformants, the lower level of GUS activity in NR-1/pGAPN2B might be the result of relatively inefficient translation or stability of the β-tubulin fusion protein.

To measure the reproducibility of GUS expression in transformants that are presumably genetically identical to D8D3,

five independent pAPGUSNNB 3′ *nor-1* integrants were assayed in duplicate for GUS activity (quantitative assay). The difference in GUS activity between duplicate samples for any one isolate did not exceed 10% of the mean. The difference between the mean of GUS activity for duplicate samples from any two isolates did not exceed 60% (GUS activity at 48 h ranged from 12 to 19 nmol of 4-methylumbelliferone/min/mg of protein).

Transformation of *A. parasiticus* CS10 with pAPGUSNP. Based on previously reported *ver-1* regulation studies (12) and the data presented above, we hypothesized that the location within the aflatoxin cluster provided a positive regulatory influence on *nor-1* promoter function under aflatoxin-inducing growth conditions. To test this hypothesis, we determined if the *nor-1* promoter would function at a different chromosomal location outside of the aflatoxin cluster. The *pyrG* locus was chosen because this selectable marker was cloned previously (20).

A. parasiticus CS10 was transformed with pAPGUSNP (Fig. 5A). Transformants (178 in total) were selected on CZ medium, transferred to aflatoxin-inducing YES agar medium, and analyzed by qualitative GUS assay, resulting in the identification of 42 GUS+ isolates. Fourteen GUS+ isolates were arbitrarily selected and screened by a rapid PCR assay for site of integration (Fig. 6). All 14 GUS+ isolates had pAPGUSNP integrated at 5′ *nor-1* (11 isolates) or 3′ *nor-1* (3 isolates) locus. The site of integration of pAPGUSNP was confirmed in four of these isolates and six additional arbitrarily selected GUS+ isolates by Southern hybridization analysis (Fig. 5). Of these 10 isolates, 7 had pAPGUSNP integrated at 5′ *nor-1* and 2 carried pAPGUSNP at 3′ *nor-1*. Isolate 57 was likely a multiple integrant of pAPGUSNP, with one copy of pAPGUSNP at both 5′ and 3′ *nor-1* (26). In contrast, of 11 isolates with pAPGUSNP integrated at *pyrG*, none expressed GUS activity.

pyrG is also expressed in *A. parasiticus* CS10 (used as the recipient) under these aflatoxin-inducing growth conditions (Fig. 7). Growth of *A. parasiticus* CS10 (*wh-1 ver-1 pyrG*) on YES medium required supplementation with uridine (100 μg/ml) while transformation of CS10 with a functional *pyrG* gene overcame this requirement, demonstrating that a functional copy of *pyrG* must be present and expressed for growth of CS10 on YES medium.

Confirmation of positional effect at *niaD*:pBNG3.0. We analyzed the function of a different *nor-1*::GUS reporter construct (pBNG3.0) (Miller et al. submitted) at *niaD* and *nor-1*. Transformation of pBNG3.0 into NR-1 (*niaD*) resulted in 117 transformants. The transformants were tested for GUS activity by the qualitative assay, resulting in 29 GUS+ colonies (25%). Of the 29 GUS+ transformants, 21 were tested for site of integration using the rapid PCR assay (Fig. 8B and C). All 21 transformants were positive for either 5′ (12 transformants) or 3′ (9 transformants) *nor-1* integration. Conversely, 22 arbitrarily selected GUS− transformants were tested for site of integration using the rapid PCR assay and all were negative for 5′ and 3′ *nor-1* integration (data not shown). Identification of 3′ integrants was important because it results in the *nor-1* promoter from the plasmid (pBNG3.0) being connected to the GUS gene while 5′ *nor-1* integration results in the chromosomal *nor-1* promoter being connected to the GUS gene. All 3′ *nor-1* integrants tested (eight isolates) were confirmed for 3′
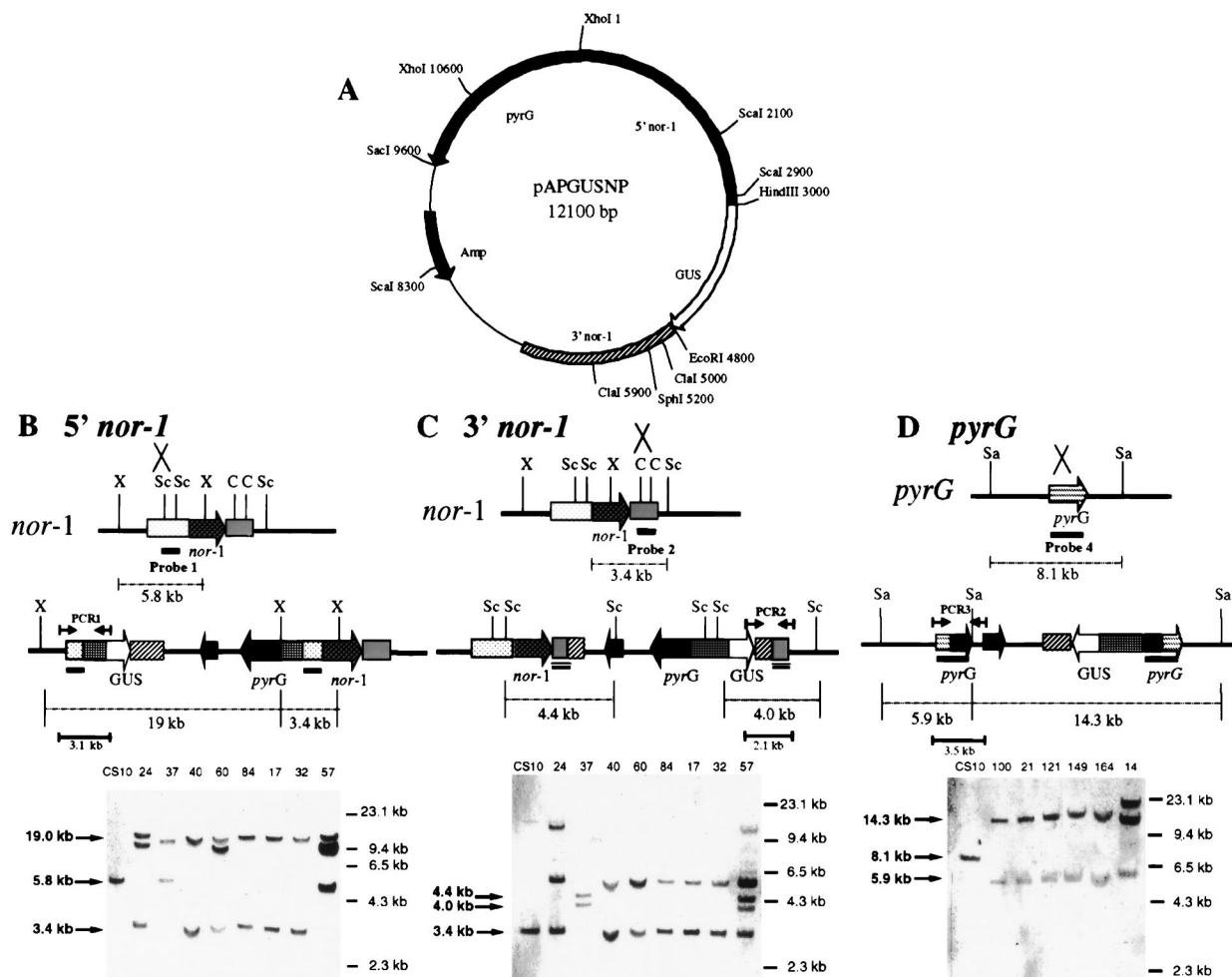
FIG. 5. Southern hybridization analysis of site of integration in pAPGUSNP transformants. (A) Restriction endonuclease map of pAPGUSNP. Only restriction endonuclease sites relevant to this study are shown. (B) Schematic for Southern hybridization and PCR analyses of pAPGUSNP integration into the 5′ region of *nor-1* and Southern hybridization data. *A. parasiticus* genomic DNA was digested with *Xho*I and probed with probe 1. Single-crossover integration of pAPGUSNP into the 5′ region of *nor-1* results in two bands, of 19 and 3.4 kb, and the disappearance of the 5.8-kb wild-type band. (C) Schematic for Southern hybridization and PCR analysis of pAPGUSNP integration into the 3′ region of *nor-1* and Southern hybridization data. Genomic DNA was digested with *Sca*I and probed with probe 2. Single-crossover integration of pAPGUSNNB into the 3′ region of *nor-1* results in two bands, of 4.0 and 4.4 kb, and the disappearance of the 3.4-kb wild-type band. (D) Schematics for Southern hybridization and PCR analysis of pAPGUSNP integration into *pyrG* and Southern hybridization data. Genomic DNA was digested with *Sac*I and probed with probe 4. Single-crossover integration of pAPGUSNP into *pyrG* results in two bands, of 14.3 and 5.9 kb, and the disappearance of the 8.1-kb wild-type band. Abbreviations: X, *Xho*I; Sc, *Sca*I; C, *Cla*I; Sal, *Sal*I. The numbers in kilobases to the right of the Southern blots in panels B, C, and D represent molecular size markers. The numbers in kilobases to the left represent the sizes of actual restriction fragments observed in the analysis.

integration by Southern hybridization analysis (data not shown). These data confirmed the results reported above for pAPGUSNNB, i.e., that integration at *nor-1* results in wild-type *nor-1* promoter activity while integration at *niaD* results in undetectable levels of *nor-1* promoter activity.

DISCUSSION

Our experiments demonstrate that the *nor-1*::GUS reporter and the wild-type *nor-1* gene are regulated in a similar manner in pAPGUSNNB transformant strain D8D3. On an aflatoxin-inducing growth medium (YES), the timing and pattern of transcript and protein accumulation driven by the *nor-1* promoter for these two genes were similar. Data obtained from

the same time course experiment also showed that the time of onset of accumulation of aflatoxin and GUS activity was the same, while the pattern of increase in aflatoxin accumulation was consistent with the pattern of increase in accumulation of GUS activity at 48 and 72 h (i.e., relatively high GUS activity preceded high levels of aflatoxin accumulation). Furthermore, when qualitative GUS assays were performed on D8D3 grown on CZ medium, a non-aflatoxin-inducing growth medium, GUS activity was not detected (data not shown). There also was a relatively low level of variation in GUS expression in transformants that were genotypically identical to D8D3 based on Southern hybridization analysis. When five arbitrarily selected isolates were cultured under identical growth conditions and then assayed for GUS activity, the difference in GUS
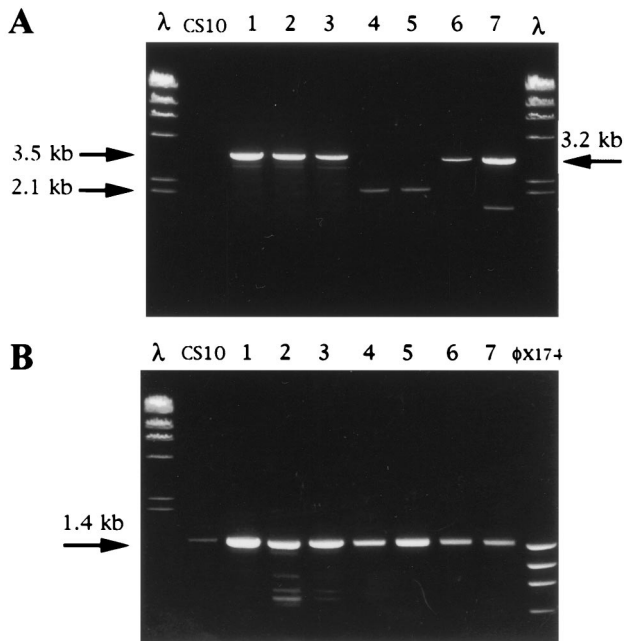
FIG. 6. PCR analysis to detect site of integration in pAPGUSNP transformants. Template DNA for 5′ and 3′ *nor-1* and *pyrG* integration assays was prepared with a rapid boiling procedure. (A) GUS⁺ and GUS⁻ transformants (lanes 1 through 7) and CS10 (negative control) analyzed for 5′ *nor-1*, 3′ *nor-1*, or *pyrG* integration. Primers PCR3 were used to detect *pyrG* integration (lanes 1, 2, and 3; GUS⁻ transformants) and generated a 3.5-kb PCR fragment. Primers PCR2 were used to detect 3′ *nor-1* integration (lanes 4 and 5; GUS⁺ transformants) and generated a 2.1-kb PCR fragment. Primers PCR1 were used to detect 5′ *nor-1* integration (lanes 6 and 7; GUS⁺ transformants) and generated a 3.1-kb PCR fragment. (B) DNA from all seven transformants and the control strain CS10 amplified with a primer pair specific for exon III of the versicolorin B synthase gene (positive control). A 1.4-kb PCR fragment was expected for all fungal isolates. Lanes containing molecular size markers in panels A and B are represented by λ *Hin*dIII digest and ϕx174 *Hae*III digest. Nucleotide sequences of all primers are found in Table 1.

activity at any time between isolates did not exceed 60%. These data validate the use of the *nor-1*::GUS reporter as a sensitive and reproducible system to monitor environmental influences on expression of this aflatoxin gene.

While *nor-1*::GUS expression at the *nor-1* locus was regulated similarly to the native *nor-1* gene, placement at two other positions (*niaD* and *pyrG*) outside of the aflatoxin gene cluster resulted in no detectable GUS activity. These data support the preliminary observation by Liang et al. (14) that chromosome location plays a role in regulation of aflatoxin gene expression. The targeting of reporter constructs to specific chromosomal locations for promoter analysis has been suggested by other investigators studying expression of fungal genes (7, 22, 25). Although the mechanisms of position-dependent gene expression have not been fully elucidated in filamentous fungi, it has been hypothesized that enhancer elements may be responsible for the position-dependent effect (12). The hypothesis that positive *cis*-acting factors have regional control over the transcription of aflatoxin genes is reasonable, since removal of aflatoxin reporter fusions from the aflatoxin gene cluster results in reduced GUS expression.

An alternative explanation for the lack of GUS activity at the

niaD and *pyrG* loci is that a transcriptionally inactive chromatin structure exists at these two sites. We think that such inactivation is unlikely for two reasons. First, NR-1 transformed with pGAPN2B (β-tubulin::GUS) expressed GUS activity at similar levels at 24, 48, and 72 h when integrated at the *niaD* locus, although the level of GUS activity in the pGAPN2B transformants was approximately 10-fold lower than those of D8D3 at 48 and 72 h. In contrast, transformant D8D4 (pAPG USNNB integrated at *niaD*), when grown under the same conditions, produced no detectable GUS activity at any of these time points (data not shown). Second, in transformants carrying pAPGUSNP, the *nor-1* promoter carried on this plasmid was not active at the *pyrG* locus even though growth on YES medium required expression of the *pyrG* gene. In addition, several pAPGUSNP transformants had no detectable GUS activity even when they carried multiple copies of the plasmid (none integrated at *nor-1*). In contrast, other multiple integrants with at least one copy of pAPGUSNP integrated at *nor-1* were GUS⁺. Based on these data and preliminary data reported by Liang et al. (14), we hypothesize that aflatoxin gene expression is influenced by an enhancer element.

The clustering, or close linking, of genes involved in the same secondary metabolic pathway is a common theme in the filamentous fungi (11). However, *cis*-acting elements that regulate many genes simultaneously in fungal gene clusters have not been identified. If an enhancer element does influence the
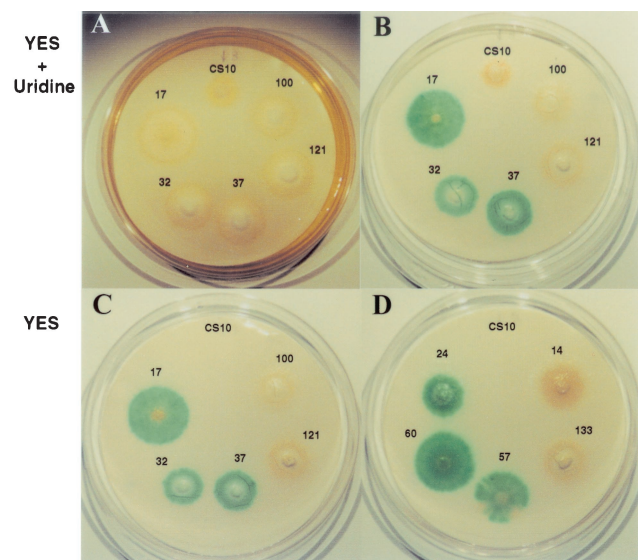

FIG. 7. Qualitative GUS activity assay on CS10 transformants harboring pAPGUSNP. The recipient strain CS10 (control) and representative *pyrG*⁺ transformants were inoculated onto nytran membranes overlaid onto YES agar medium (C and D) or YES supplemented with 5 mg of uridine per plate (A and B). A qualitative GUS assay was conducted on filters for panels B, C, and D. Transformants carried a single copy of pAPGUSNP integrated at 5′ *nor-1* (transformants 17 and 32), 3′ *nor-1* (transformant 37), or *pyrG* (transformants 100 and 121). Transformants 24 and 60 carried multiple copies of pAPGUSNP with at least one copy integrated at 5′ *nor-1*. Transformant 57 carried multiple copies of pAPGUSNP with at least one copy integrated at 3′ *nor-1*. Transformants 14 and 133 carried multiple copies of pAPGUSNP with at least one copy at *pyrG* and no copies at *nor-1*.
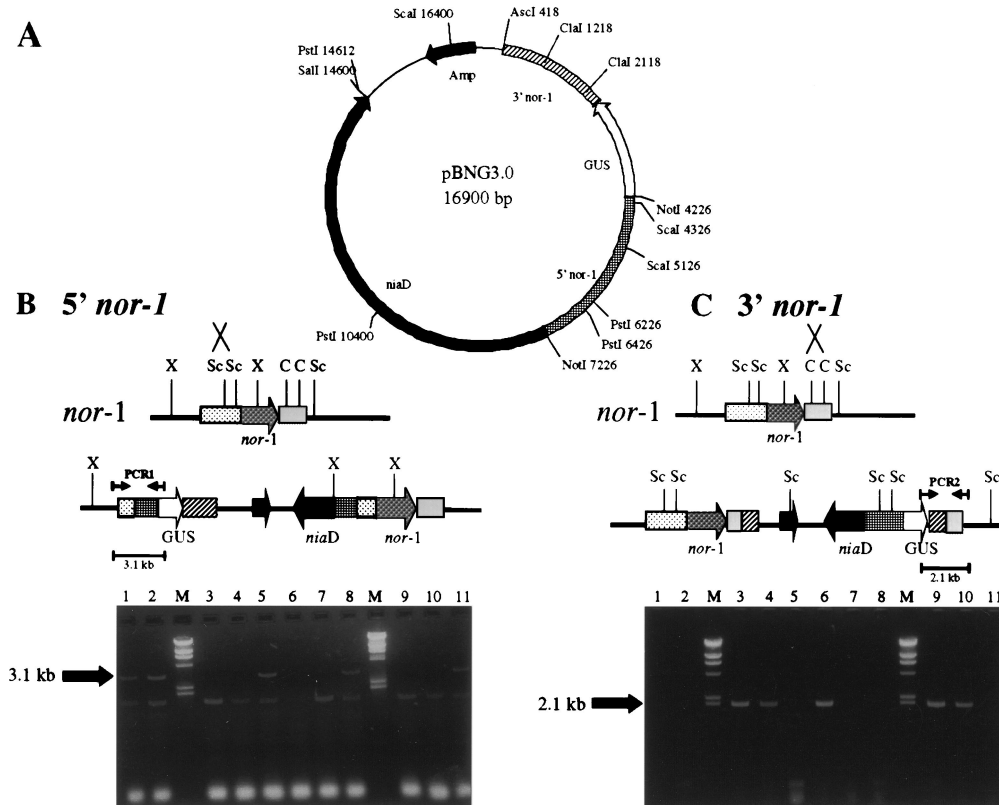
FIG. 8. PCR analysis of site of integration in pBNG3.0 transformants. (A) Restriction endonuclease map of pBNG3.0. Only restriction endonuclease sites relevant to this study are shown. Template DNA was prepared using a rapid boiling procedure. Ten GUS$^+$ transformants (lanes 1 to 6 and 8 to 11) and one GUS$^-$ transformant (lane 7) were analyzed for both 5' and 3' nor-1 integration. (B) Schematic for 5' nor-1 integration with PCR data. 5' nor-1 integrants resulted in a 3.1-kb PCR fragment (lanes 1, 2, 5, 8, 11) with PCR1 primers. (C) Schematic for 3' nor-1 integration with PCR data. 3' nor-1 integrants resulted in a 2.1-kb PCR fragment (lanes 3, 4, 6, 9, 10) with PCR2 primers. All GUS$^+$ transformants were 5' or 3' nor-1 integrants while the GUS$^-$ transformant (lane 7) was negative for both 5' (9B) and 3' (9C) nor-1 integration. Lanes labeled M in panels B and C represent molecular size markers.

regulation of nor-1 transcription, it is located at least 3 kb upstream from the transcription initiation site in the 5' nor-1 region, at least 1.8 kb downstream of the transcription termination site in the 3' region of nor-1, or within the nor-1 coding region. Because of similar position-dependent expression observed with the ver-1::GUS reporter construct (14), it is also possible that the same cis-acting element is influencing the regulation of both nor-1 and ver-1. Studies performed on the SpoC1 gene cluster in A. nidulans showed that clustered genes can be coordinately expressed during development and that placement of cluster genes at ectopic chromosomal locations results in the loss of that coordination (15, 21). The physical linkage of aflatoxin biosynthesis genes also may have a regulatory role. Studies designed to establish such a phenomenon and to determine the nature of the regulatory mechanism may eventually lead to a broader understanding of the expression of secondary metabolism genes and the ability to manipulate its expression in microorganisms.

We developed a rapid procedure to prepare DNA templates from fungal colonies that are sufficiently pure for PCR analysis of the site of plasmid integration into the A. parasiticus genome. This development was critical for current and future studies on the regulation of the nor-1 and ver-1 promoters because detection of the correct site of integration in the ge-

nome is essential to accurately measure environmental influences on the aflatoxin gene promoters. In this regard, the speed and effectiveness of the rapid assay make screening of large numbers of fungal transformants practical.

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