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Regulated Expression of the *nor-1* and *ver-1* Genes Associated with Aflatoxin Biosynthesis

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RNA transcript accumulation for the *ver-1* and *nor-1* genes, which are associated with aflatoxin biosynthesis in the fungus *Aspergillus parasiticus*, was measured before and during aflatoxin production in liquid shake culture. Transcripts were not detected until near the end of trophophase (growth phase) and could still be observed well into stationary phase during batch fermentation in an aflatoxin-supporting growth medium. Maximum accumulation of both transcripts occurred just prior to the onset of stationary phase. Aflatoxin B_1 was first detected approximately 8 h after the appearance of the *ver-1* and *nor-1* transcripts. In contrast, maximum transcript accumulation for the *pyrG* gene (encoding orotidine monophosphate decarboxylase), which is involved in primary metabolism, was observed at the onset of trophophase when the *ver-1* and *nor-1* transcripts could not be detected. Accumulation of the *ver-1* and *nor-1* transcripts was also studied following a nutritional shift from a non-aflatoxin-supporting medium (peptone mineral salts [PMS]) to a glucose-containing medium (glucose mineral salts [GMS]), which does support aflatoxin biosynthesis. Transcripts from *ver-1* and *nor-1* could not be detected on PMS medium but did accumulate approximately 4 to 7 h following transfer to GMS medium. Additionally, aflatoxins were not detected in PMS medium but were observed to accumulate within 24 h after the shift from PMS to GMS. These data suggest that aflatoxin biosynthesis is in part regulated by the accumulation of the *ver-1* and *nor-1* transcripts.

Aflatoxins are a group of polyketide-derived secondary metabolites produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Recognition of the hepatocarcinogenic and toxic effects of aflatoxins prompted investigations to understand the production of this common food contaminant (11, 21). These efforts have allowed the proposal of a generally accepted, but incomplete, biosynthetic pathway (5, 18, 22). Additionally, numerous enzymes involved in the bioconversion of aflatoxin intermediates have been identified and purified to different degrees (3, 4, 13, 14, 16, 20, 22, 32, 33). The introduction of molecular biology techniques into the study of toxigenic fungi has recently permitted the isolation of several genes associated with aflatoxin biosynthesis (10, 26, 30). Emphasis can now be focused on identifying the molecular mechanisms which regulate aflatoxin production.

Aflatoxins are typically produced during idiophase, the period when logarithmic growth has ceased and secondary metabolites are formed. The activities of certain enzymes involved in aflatoxin biosynthesis are not detected until this time (13, 15), and the appearance and activity of at least one of these proteins require de novo protein synthesis (15). However, the molecular mechanisms that regulate the induction of aflatoxin-biosynthetic enzymes still remain unclear.

The purpose of this study was to better understand the regulation of aflatoxin biosynthesis in the hope of eventually defining critical points in the pathway which may serve as possible target sites for the inhibition of aflatoxin biosynthesis. The accumulation of RNA transcripts for genes associated with the conversion of the aflatoxin intermediate norsolorinic acid to averantin or averufanin (the *nor-1* gene) and the aflatoxin intermediate versicolorin A to sterigmatocystin (the *ver-1* gene) was studied before and during aflatoxin production. Evidence that RNA transcripts for these genes do not accumulate until near the end of trophophase and that this pattern of transcript accumulation may play a direct role in the regulation of aflatoxin biosynthesis is presented.

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MATERIALS AND METHODS

Fungal strains and culture conditions. *A. parasiticus* NRRL 5862 (SU-1) served as the aflatoxin-producing wild-type strain and was maintained on potato dextrose agar.

Batch fermentation analysis. Frozen (−80°C in 15% glycerol) conidia (2×10^6) of *A. parasiticus* NRRL 5862 were inoculated into 250-ml silane-treated Erlenmeyer flasks, each containing 100 ml of defined minimal medium (AM) (1). Five glass beads (diameter, 3 to 4 mm) were included in each flask to aid in aeration of the culture and to facilitate dispersed mycelial growth (28). Mycelial growth was visibly more uniform among duplicate flasks containing glass beads, since they prevented the inconsistent aggregation or clumping of the mycelium that is typically observed in *Aspergillus* shake cultures without beads. All flasks were incubated in the absence of light at 29°C in an orbital shaker with shaking at 220 rpm. Triplicate flasks were removed at each time point and analyzed individually for mycelial dry weight, pH of the medium, and aflatoxin concentration in the filtrate. For each time point and each measurement, the average value for the three flasks and the standard error were then calculated. Additional flasks were included for RNA isolation, beginning at 24 h and continuing every 12 h up to 146 h after inoculation.

Nutritional shift assay. A modified nutritional shift protocol (8, 9) was employed to determine when the *nor-1* and *ver-1* RNA transcripts accumulate relative to a shift from non-aflatoxin-supporting conditions to those which support aflatoxin biosynthesis. Three silane-treated Fernbach flasks,

each containing 500 ml of peptone mineral salts (PMS) medium (9), were each inoculated with 2.5×10^7 conidia of *A. parasiticus* NRRL 5862 (from a frozen stock) and grown for 65 h at 29°C in an orbital shaker with shaking at 200 rpm. PMS medium is similar to AM medium (1) except that in PMS medium, glucose is replaced by peptone, which serves as the sole carbon source. This medium does not support or induce aflatoxin biosynthesis (9). The mycelium from all three flasks was combined, harvested by filtration through cheesecloth, and distributed (5 g [wet weight] of mycelium per flask) to 250-ml silane-treated flasks containing 30 ml of either PMS or glucose mineral salts (GMS) medium (8). GMS, which is capable of inducing and supporting aflatoxin biosynthesis, is identical to PMS except that peptone (6%) is replaced by glucose (6%). After the shift, incubation of the cultures was continued for up to 48 h under the same conditions. One PMS- and one GMS-containing flask was removed at each time point so that the mycelium could be harvested and used for RNA extraction. Filtrates from each time point were saved for quantitative analysis of aflatoxin B_1.

Analysis of mycelial dry weight and aflatoxin concentration. Fungal cultures were removed from the incubator-shaker at specified times, and the mycelium was harvested by filtration through Miracloth (Behring Diagnostics, La Jolla, Calif.). The mycelium collected from each culture flask was dried completely at 70°C prior to weighing. A sample of each filtrate was tested for pH, and the remainder was used for determination of aflatoxin production. Only the filtrate was tested, since both aflatoxin B_1 and aflatoxin G_1 are known to efficiently pass through the mycelial wall at 29°C (23). Aflatoxin B_1 was analyzed by using direct competitive enzyme-linked immunosorbent assay (ELISA) according to the method of Pestka (27). Aflatoxin B_1 monoclonal antibodies (kindly provided by J. Pestka, Michigan State University) and aflatoxin B_1-horseradish peroxidase conjugate, prepared by the methods of Chu et al. (12), were used in this study. The aflatoxin B_1 monoclonal antibody was able to detect a minimum of 1 ng of aflatoxin B_1 per ml of growth medium.

Isolation and analysis of RNA. Fungal mycelium was collected by filtration through Miracloth and then quickly frozen in liquid nitrogen. Total RNA was then isolated and purified by using a hot-phenol protocol (2). Northern (RNA) analysis of RNA samples was performed as described by Maniatis et al. (24). Approximately 7 μg of total RNA per sample was separated by electrophoresis in a 1.2% denaturing formaldehyde agarose gel and then transferred by capillary action to a Nytran membrane (Schleicher and Schuell, Inc., Keene, N.H.), which served as a solid support. Radiolabelled DNA probes were generated with [^{32}P]dCTP (New England Nuclear) with the Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemicals) according to the manufacturer's instructions. A 0.6-kb *Ava*I-*Bam*HI restriction endonuclease fragment of an internal portion of the *A. parasiticus ver-1* gene (GenBank accession no. M91369, a 1.5-kb *Bgl*II-*Cla*I fragment containing the *A. parasiticus nor-1* gene (10), and a 2.8-kb *Bam*HI-*Sal*I fragment containing the *A. parasiticus pyrG* gene (31) were used as DNA probes for Northern analysis.

Chemicals. Chemicals, unless specifically referenced in the text, were purchased from Sigma Chemical Company. Potato dextrose agar was purchased from Difco Laboratories (Detroit, Mich.).

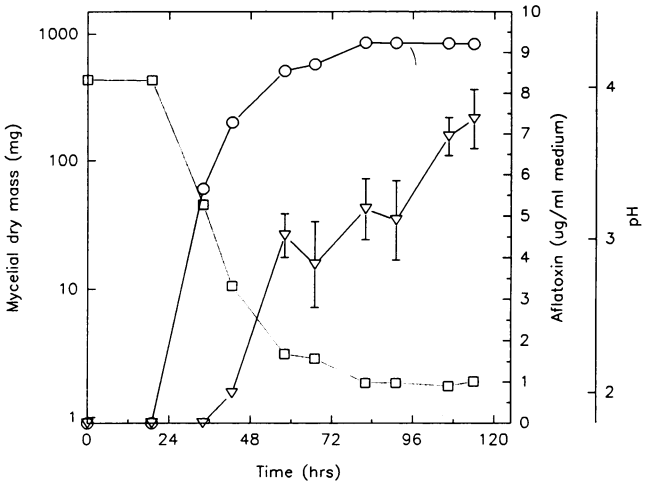

FIG. 1. Production of aflatoxin during batch fermentation. *A. parasiticus* NRRL 5862 was inoculated into defined medium (time zero) and grown at 29°C under shake conditions. Triplicate samples were removed at each time point and analyzed for mycelial dry mass (○), pH (□), and aflatoxin B_1 (in micrograms per milliliter of medium) (▽). Vertical bars, standard error.

RESULTS

Expression of aflatoxin-associated genes during batch fermentation. Wild-type *A. parasiticus* NRRL 5862 was grown in AM medium under conditions which support aflatoxin biosynthesis. The patterns of aflatoxin accumulation and mycelial growth (Fig. 1) correlated with patterns observed in previous growth studies involving aflatoxigenic *A. parasiticus* strains (29). Aflatoxin B_1 was first detected near the end of trophophase (growth phase), approximately 42 h after inoculation. The onset of trophophase was accompanied by a rapid decrease in the pH of the medium. The concentration of accumulated aflatoxin B_1 in the growth medium increased rapidly well into stationary phase, with the greatest rate of accumulation occurring between 42 and 58 h after inoculation. The onset of stationary phase (at approximately 60 h) was accompanied by a brief plateau in aflatoxin accumulation before the concentration increased again at approximately 96 h. The level of aflatoxin did decrease slightly between 120 and 150 h (data not shown).

The error rate for pH and mycelial mass was negligible between duplicate samples in the continuous-growth study. However, aflatoxin accumulation in the filtrate was somewhat variable. This inconsistency may be explained by variations in the efficiency of aflatoxin B_1 transport out of the cell, and it probably could have been partly eliminated by analyzing total aflatoxin B_1 (mycelium and filtrate). A period of decreased aflatoxin accumulation noted between 58 and 91 h is believed to be a result of the inoculum size. Such a biphasic pattern of aflatoxin accumulation has been reported to occur when the initial spore concentration is greater than 10^3 conidia per ml of medium (29).

Northern hybridization analysis with *ver-1* and *nor-1* DNA probes was done with total RNA purified from mycelial cultures harvested at various time points during batch fermentation. The sizes of the transcripts detected during this analysis were determined to coincide with previously published results (10, 30) of approximately 1.0 and 1.3 kb for the *ver-1* and *nor-1* transcripts, respectively. RNA transcripts from each gene were not detected until 36 h after inoculation

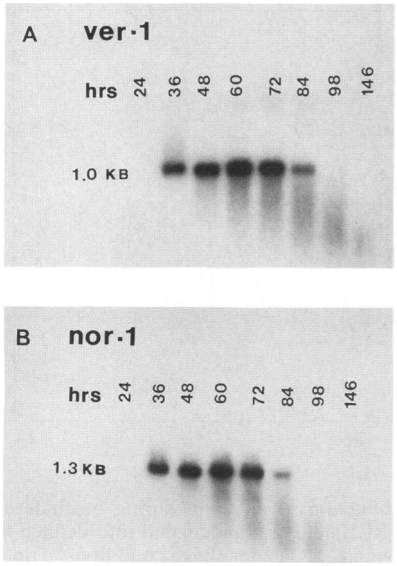

FIG. 2. Accumulation of *ver-1* (A) and *nor-1* (B) transcripts during batch fermentation. Approximately 7 μg (per sample) of total RNA isolated at various time points during the growth study was separated by denaturing gel electrophoresis. RNA was transferred to Nytran membranes and then hybridized to ^{32}P-labelled probes containing either the *ver-1* or *nor-1* gene.

(Fig. 2). This was approximately 8 h before the first observation of aflatoxin B_1 in the filtrate. Maximum accumulation of these transcripts occurred between 60 and 72 h after inoculation. Evidence for transcript degradation, indicated by smearing below the band, first appeared at 48 h and increased thereafter. Degradation products from the *ver-1* and *nor-1* transcripts could still be detected as late as 146 h after inoculation.

The accumulation of RNA transcribed from the *pyrG* gene, which encodes an orotidine monophosphate decarboxylase involved in uridine biosynthesis, was also examined. This analysis was used to compare the levels of RNA transcribed from genes associated with aflatoxin biosynthesis with the levels of RNA transcribed from a gene involved in primary metabolism. The highest level of the *pyrG* transcript was detected at the 24-h time point, corresponding to the period of the greatest rate of mycelial-mass production (Fig. 3). *pyrG* transcript accumulation decreased after 36 h, and the transcript could not be detected beyond the onset of stationary phase (approximately 84 h after inoculation). The *pyrG* transcript revealed little degradation, especially at the early time points, confirming that the absence of the *ver-1* and *nor-1* transcripts at 24 h was not a result of the quality of the RNA preparation.

Expression of aflatoxin-associated genes following nutritional shift. The *ver-1* and *nor-1* transcripts were not detected in RNA isolated from the mycelium initially grown in PMS medium (Fig. 4). Likewise, the *ver-1* and *nor-1* transcripts could not be detected in RNA isolated from mycelium after transfer to fresh PMS medium. However, these transcripts were observed 7 h after transfer to GMS medium. The *pyrG* transcript was detected in very small amounts in all of the RNA samples tested (data not shown), suggesting that the absence of detectable *ver-1* and *nor-1* transcripts was not due to a lack of RNA quality. The low level of the *pyrG* transcript is presumably a result of limited growth following

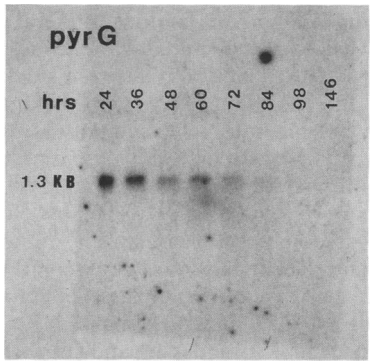

FIG. 3. Accumulation of *pyrG* transcript during batch fermentation. Northern analysis was performed as described for Fig. 2, except that a *pyrG*-containing DNA fragment was used to prepare the ^{32}P-labelled probe.

transfer of mycelium that was harvested during the stationary phase of growth. Direct competitive ELISA analysis detected aflatoxin B_1 in the medium (0.6 μg/ml of filtrate) 24 h after transfer from PMS medium to fresh GMS medium. The quantity of aflatoxin B_1 in the GMS medium increased approximately sixfold (3.7 μg/ml of filtrate) by 48 h whereas no aflatoxins could be detected even 48 h after transfer from the original PMS medium to the fresh PMS medium.

DISCUSSION

During batch fermentation of *A. parasiticus*, the *ver-1* and *nor-1* RNA transcripts were observed to accumulate in a manner similar to what might be expected of genes involved in secondary metabolism (17). RNA transcribed from these genes was not detected until near the end of trophophase, and this accumulation was followed approximately 8 h later by idiophase, when aflatoxins are produced. The pattern of

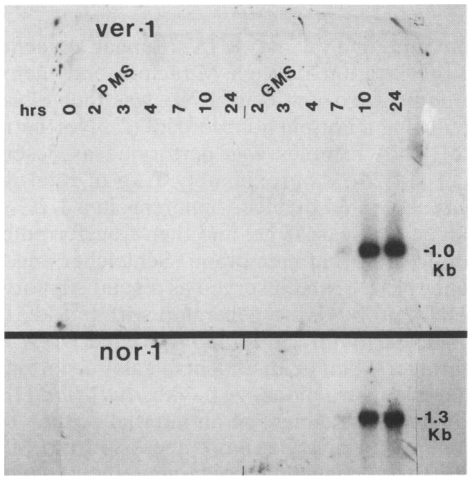

FIG. 4. Accumulation of *ver-1* and *nor-1* transcripts following nutritional shift. *A. parasiticus* NRRL 5862 grown for 65 h in PMS medium was equally distributed to fresh PMS and GMS media, and growth was continued under the same incubation conditions. RNA was isolated before transfer (time zero) and at various times following transfer from the spent PMS medium to fresh GMS and PMS media. Approximately 7 μg of total RNA (per sample) was analyzed as described for Fig. 2.

accumulation of the *pyrG* transcript, which is involved in primary metabolism, differed significantly from the patterns of accumulation of the *ver-1* and *nor-1* transcripts. Maximum accumulation of the *pyrG* transcript occurred during trophophase (approximately 24 h after inoculation), a period when the *ver-1* and *nor-1* transcripts were not detectable, whereas maximum accumulation of the *ver-1* and *nor-1* transcripts was observed just prior to the onset of stationary phase (approximately 60 to 72 h after inoculation). Additionally, the presence of the *ver-1* and *nor-1* transcripts continued well beyond the time when the *pyrG* gene was observed.

The regulatory mechanisms involved in the induction of aflatoxin biosynthesis are not fully understood. By using a nutritional shift study, Buchanan et al. (8) demonstrated that a shift from a peptone-containing medium to a glucose-containing medium induced aflatoxin biosynthesis in *A. parasiticus* approximately 10 h after the shift. The addition of translation and transcription inhibitors at various times after the nutritional shift suggested that de novo RNA synthesis and protein synthesis must occur for the induction of aflatoxin biosynthesis. The time period which Buchanan et al. observed for the required RNA synthesis (3 to 6 h postshift) is consistent with the timing of the appearance of the *ver-1* and *nor-1* transcripts (7 h postshift) observed in the current study, in which a similar nutritional shift format was used. The factors which trigger *nor-1* and *ver-1* transcript accumulation in *A. parasiticus* are not known, although previous studies implicate carbon catabolite induction as one potential mechanism. Glucose is reported to repress tricarboxylic acid cycle activity in *A. parasiticus* (7), which may contribute to a lowering of the intracellular NADPH/NADP ratio (9). A decrease in the NADPH/NADP ratio has been hypothesized to play a role in the induction of aflatoxin biosynthesis, although this connection is still tenuous (25).

The timing of the appearance of the *ver-1* and *nor-1* transcripts relative to aflatoxin detection in batch fermentation and nutritional shift analysis strongly suggests that aflatoxin biosynthesis is partly regulated by the accumulation of the *ver-1* and *nor-1* RNA transcripts. It is generally believed that genes of the filamentous fungi are usually regulated at the transcriptional level (19). We speculate that the observed rate of RNA accumulation parallels the rate of RNA transcription for the aflatoxin genes examined. However, it will not be possible to conclusively determine whether this accumulation is controlled primarily by the rate of RNA transcription or by mechanisms affecting RNA stability until these processes are measured directly.

Sequence analysis of the *ver-1* gene suggests that it is a structural gene encoding a dehydrogenase (30). We predict that the *nor-1* gene is also a structural gene because of its implied function in the conversion of norsolorinic acid to averantin or averufanin and the presence of a NADP binding motif in the sequence (unpublished data). The similarities in timing and levels of expression between these genes might indicate that they are controlled by common regulatory factors. Supporting this hypothesis is the observation of significant sequence identity between the upstream promoter regions of these genes. The 5′ regions of both the *ver-1* and *nor-1* genes are currently being investigated by means of reporter gene fusions and electrophoretic mobility shift assays to help identify the regulatory proteins involved in aflatoxin biosynthesis.

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ADDENDUM

Studies on the accumulation of the *nor-1* transcript in two other aflatoxin-supporting growth media, potato dextrose broth plus 2% yeast extract and the defined medium of Reddy et al., containing sucrose as the carbon source (27a), have now been completed. The *nor-1* transcript was observed to accumulate in these media in a pattern similar to that observed in AM medium, although the absolute times of accumulation and the maximum dry weights and levels of aflatoxin varied among the three media. One important difference was observed with Reddy medium. The level of the *nor-1* transcript did not decline after reaching stationary phase and aflatoxin continued to accumulate during this time, lending further support to the hypothesis that the level of the *nor-1* transcript in part regulates aflatoxin biosynthesis.

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