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Molecular Cloning of Genes Related to Aflatoxin Biosynthesis by Differential Screening

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A differential hybridization strategy was used to clone genes associated with aflatoxin biosynthesis. A genomic library, formed between nuclear DNA and the pUC19 plasmid, was screened with three different cDNA probes by the colony hybridization procedure. Nineteen clones were selected; all were positively correlated with and presumably enriched with genes associated with aflatoxin production. Some of these clones were further characterized by using them as probes in Northern (RNA blot) hybridizations. Five clones hybridized strongly with some polyadenylated RNAs formed during the transition to or during idiophase when aflatoxin was produced. However, little or no corresponding hybridization occurred with polyadenylated RNAs formed in early and mid-log growth phase. Two of the clones were further used as probes to hybridize with polyadenylated RNAs formed under aflatoxin-permissive and nonpermissive temperatures. Hybridization occurred with RNA species formed under the permissive temperature only.

Aflatoxins are polyketide-derived, toxic secondary metabolites produced by certain strains of *Aspergillus parasiticus* and *A. flavus* (5, 14). As is characteristic of secondary metabolism, the aflatoxin-biosynthetic pathway is not essential for growth (8). Little is known about the genetic basis of aflatoxin production versus nonproduction, largely because of the asexual nature of these *Aspergillus* species. The recent advent of molecular cloning procedures, however, has provided a means to study the mechanisms of gene expression in these fungi and to further elucidate the aflatoxin pathway.

To date, no aflatoxin-related genes have been cloned, and molecular tools are only beginning to be applied to the aflatoxigenic fungi (25). In the present study, we report a differential molecular screening procedure that has generated DNA clones enriched for aflatoxin-related activities.

There is evidence that some of the genes responsible for aflatoxin biosynthesis are differentially expressed under certain culture conditions related to growth and nutrition (1, 11, 12, 14, 19, 24). Similar to other secondary metabolites, aflatoxin is produced at the beginning and during the idiophase of culture growth (14). Two enzymes in the aflatoxin pathway, methyltransferase and oxidoreductase, are produced de novo during the transition to idiophase in *A. parasiticus* (11, 12).

Aflatoxin production is influenced at the level of transcription by the presence of readily available carbon sources in the culture medium. The presence of sugar in the medium, for example, supported aflatoxin production, but in the absence of sugar no aflatoxin was produced (1). When aflatoxigenic cultures were shifted from such nonsupporting to supporting media, aflatoxin was produced following a characteristic lag phase (1, 2). The addition of dactinomycin to the supporting medium, however, inhibited aflatoxin formation, suggesting that de novo gene activity was necessary for its production.

Culture temperature is also known to influence aflatoxin formation at the transcription level. The temperature range for aflatoxin production has been reported to be between 18 and 35°C (24). In a recent experiment, Niehaus (19) found that when *A. parasiticus* was shifted from a nonpermissive (37°C) to a permissive (24°C) temperature, versicolorin, an intermediate in the aflatoxin pathway, was produced after a 6-h lag. Such induction was blocked by addition of dactinomycin to the culture medium at the time of the temperature shift, suggesting again that de novo gene activity is a prerequisite for aflatoxin formation.

All three factors described above—growth phase, carbon source, and temperature—appear to induce differential gene expression prior to aflatoxin formation. We used all three of these conditions in our cloning strategy, which generated 19 clones enriched for genes related to aflatoxin biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions. *A. parasiticus* NRRL 2999 was the principal strain used in this study and is well documented for aflatoxin B1 production (1, 2). The media used have been reported before (1, 2). Nonsugar, peptone minimal salts (PMS) medium was used as the aflatoxin-nonpermissive medium, in which no detectable aflatoxin was produced. Maltose minimal salts (MMS) medium was used as the aflatoxin-permissive medium, in which a high quantity of aflatoxin was produced. MMS medium is the same as PMS medium except that peptone is replaced by maltose.

Before an experiment was initiated, spores maintained at −30°C in 15% glycerol were inoculated on solid sporulation medium (21) and incubated at 35°C for 1 week. Fresh spores were harvested in 0.01% Tween 80, washed with sterile water, and inoculated into liquid media at 5×10^5 spores per ml. The mycelium was grown in 500 ml of medium in a 2-liter Erlenmeyer flask on a shaker (240 rpm) at 26°C, harvested, filtered through Miracloth, washed with TE buffer (10 mM Tris-Cl, 1 mM EDTA), frozen in liquid nitrogen, and stored at −70°C until used for DNA and RNA extraction.

In media and temperature shift experiments, spores were inoculated into liquid PMS medium and cultured for 48 h before the mycelium was harvested by filtering through Miracloth. The mycelium was subsequently washed with

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sterile water, homogenized in a Waring blender at maximum speed for 1 min, filtered through Miracloth, and subdivided. Each aliquot was transferred to a flask containing fresh PMS or MMS medium, cultured at a designated temperature, and harvested at a designated time.

Aflatoxin assay. A direct competitive enzyme-linked immunosorbent assay (ELISA) was used to determine the amount of aflatoxin B1 in the medium (9).

DNA isolation. Five grams of late-log-phase mycelium was ground to a fine powder in liquid nitrogen and homogenized in 10 ml of TEN buffer (50 mM Tris-Cl [pH 8.0], 10 mM EDTA, 150 mM NaCl). After sodium dodecyl sulfate (SDS) was added to a concentration of 1%, the mixture was incubated at 60°C for 15 min, cooled on ice, and extracted successively with phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). The DNA was precipitated by adding NaCl to 0.15 M and 2 volumes of cold ethanol, treated with 50 μg of RNase A per ml, and extracted and precipitated again as above.

Plasmid isolation was done by the alkali lysis procedure (23). Samples were further treated with RNase A and extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). For large-scale preparations, the plasmid was purified by CsCl centrifugation (23).

Establishment of a genomic DNA library. Genomic DNA was partially digested with *Sau*3A and fractionated by gel electrophoresis, and fragments of 5 to 7 kb were recovered from the agarose gel and purified with an Elutip-D column (Schleicher & Schuell, Keene, N.H.). The pUC19 plasmid (GIBCO BRL, Grand Island, N.Y.) was made linear with *Bam*HI, dephosphorylated with calf intestinal alkaline phosphatase (GIBCO BRL), and purified by agarose gel and an Elutip-D column. After the pooled DNA was ligated to the plasmid, the mixture was used to transform *Escherichia coli* DH5 (GIBCO BRL). The transformants were spread directly on nylon membranes, cultured on LB (Luria-Bertani [23]) plates containing 100 μg of ampicillin per ml (LB-Amp), and allowed to form colonies. Approximately 20,000 transformants were obtained. We randomly tested 20 colonies for uniformity of DNA insertion, and 19 were found to contain DNA inserts with an average size of 5.5 kb. A total of 1.04×10^8 bp of the random DNA fragments were cloned, which represents a >95% probability that all the genome fragments were cloned at least one time, assuming that the size of the genome approximated 2.7×10^7 bp, as found in *A. nidulans* (29).

The membrane-grown colonies were replica-plated to other membranes for colony hybridization. The original set was plated on LB-Amp medium containing 15% glycerol and stored at −20°C.

The colonies selected from the first cycle of differential screening were isolated with toothpicks, spotted on four sets of LB-Amp plates, allowed to form colonies, and transferred to nylon membranes. One set was stored as a sublibrary, and the other three sets were used in the second cycle of differential screening.

Poly(A)$^+$ RNA isolation and ^{32}P-labeled cDNA preparation. Total RNA was extracted from mycelium by the method of Timberlake (28), and polyadenylated [poly(A)$^+$] RNA was isolated with an oligo(dT)-cellulose column (3). ^{32}P-labeled cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Bio-Rad Laboratories, Hercules, Calif.), with a specific activity of 10^8 cpm/μg of DNA.

Colony hybridization. The colonies on nylon membranes were lysed, treated, and blotted as suggested earlier (23). The hybridizations were carried out at 68°C in an aqueous solution containing 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 0.2% SDS, and 100 μg of single-stranded salmon sperm DNA per ml. The washing stringency for the hybridizations was 0.1× SSC at 68°C for 1 h (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

In the first cycle of differential hybridization, the membranes were successively hybridized with three different cDNA probes.

(i) PMS(−). The labeled cDNA was transcribed from poly(A)$^+$ RNA isolated from mycelium grown in nonpermissive PMS medium for 48 h, followed by a shift to fresh PMS for an additional 9 h. No aflatoxin was detectable in this medium at any time.

(ii) MMS(E). The labeled cDNA was transcribed from poly(A)$^+$ RNA isolated from an early-log-phase culture when the fresh weight of the mycelium accounted for only 10% of the maximum fresh weight. These cultures were developed from spores inoculated directly into MMS medium and incubated for 18 h. Replicate cultures in MMS were used to measure the kinetics of aflatoxin synthesis. Aflatoxin was produced in this medium 30 h after spore inoculation.

(iii) MMS(+). The labeled cDNA was transcribed from poly(A)$^+$ RNA isolated from mycelium grown in PMS for 48 h and then in MMS for 9 h. Aflatoxin biosynthesis was initiated in the established mycelium beginning at 9 h in MMS medium and increased sharply thereafter.

Following hybridization with a probe, membranes were kept moist in plastic bags and exposed to X-ray film for 2 to 4 days at −70°C. Before the membranes were exposed to another probe, they were washed in 0.4 M NaOH on a shaker at 42°C for 30 min and washed further in 0.1× SSC–0.5% SDS–0.2 M Tris-Cl (pH 7.5) on a shaker at 42°C for another 30 min.

In the second cycle of differential hybridization, each of the triplet sets of membranes that housed the selected colonies were hybridized with probes PMS(−), MMS(E), and MMS(+) under the same conditions so that the results could be compared.

Northern (RNA blot) hybridization. Ten micrograms of denatured poly(A)$^+$ RNA was loaded into each lane in a 1.2% agarose gel and run in the presence of formaldehyde (3). Following electrophoresis, the gel was washed with diethylpyrocarbonate-treated water, soaked in 25 mM NaOH for 20 min, rinsed with water, immersed in 20× SSC for 40 min, and capillary-transferred onto a nylon membrane with 20× SSC solution overnight. The membrane was baked at 80°C for 1.5 h in a vacuum oven and hybridized at 62°C in an aqueous solution (23) with a probe constructed from the recombinant plasmid by the Prime-a-gene random primer labeling system (Promega, Madison, Wis.). The washing stringency was 1× SSC–0.1% SDS at 62°C for 30 min.

RESULTS

Differential screening of the genomic DNA library. The three labeled cDNA probes, PMS(−), MMS(E), and MMS(+), were each hybridized successively to the same membranes hosting the DNA library, and each was found to hybridize to approximately 60% of the colonies. When the X-ray films were compared, about 0.6% of the 20,000 colonies were found to be differentially hybridized by the three probes. These distinctive colonies, and a few closely associated colonies, were isolated and formed a sublibrary of

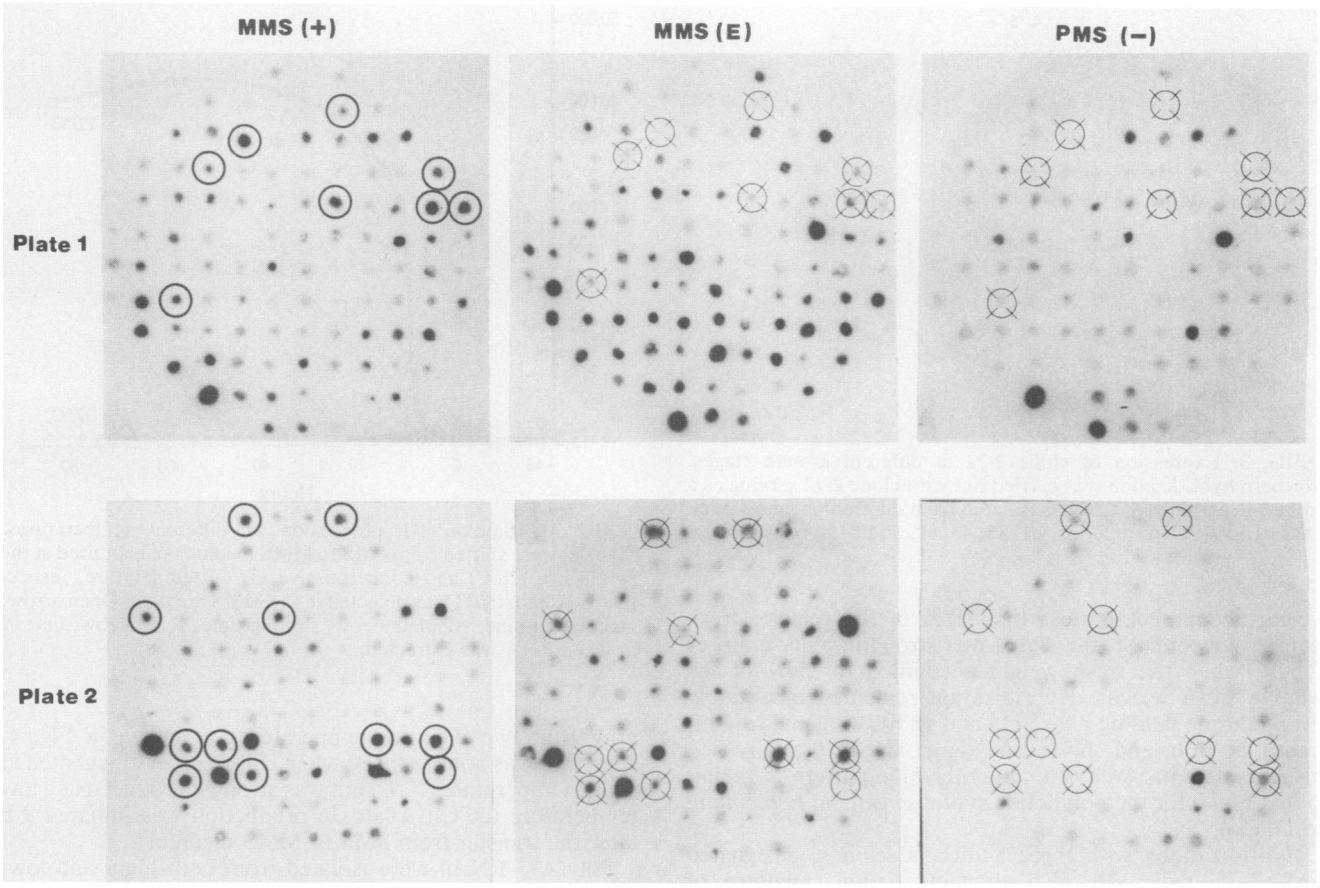

FIG. 1. Differential hybridization. The candidate colonies on plates 1 and 2 were selected from the first cycle of screening. Triplicates of each plate were hybridized a second time with the same three probes, MMS(+), MMS(E), and PMS(−). The circled colonies represent presumptive aflatoxin-related genes, which hybridized strongly with MMS(+) but weakly or not at all with MMS(E) or PMS(−).

about 200 so-called candidate colonies. All of these colonies were subsequently screened by a second cycle of hybridizations (Fig. 1). Nineteen colonies (clones) were found to differentially hybridize with the three probes. There was strong hybridization with the MMS(+) probe, weak hybridization with the MMS(E) probe, and very weak hybridization with the PMS(−) probe with only three clones showing a faint signal. When the plasmid DNAs from these clones were digested with *Eco*RI and *Hin*dIII, most showed an insert of 4.5 to 7 kb, with an average size of 5.5 kb.

Expression of the cloned aflatoxin-related sequences at different growth stages. The relationship between growth (mycelial fresh weight) and aflatoxin production was determined and is shown in Fig. 2. The fresh weight increased rapidly between 20 and 24 h (log phase) but slowed thereafter. Aflatoxin B1 levels increased logarithmically after the mycelium had attained 54% relative fresh weight (24 h) and continued to increase during late log phase and through stationary phase.

Poly(A)$^+$ RNAs were isolated from the above-mentioned cultures at 20, 22, 24, 36, 48, 60, and 84 h, and these age- and growth phase-related RNAs were used in Northern hybridization with probes prepared from some of the 19 candidate clones. An approximately 7-kb band was detected by one of the clones, designated 2-72, and the band showed developmental regulation (Fig. 3). The hybridization signal was weak in young cultures (less than 22 h), when the fresh

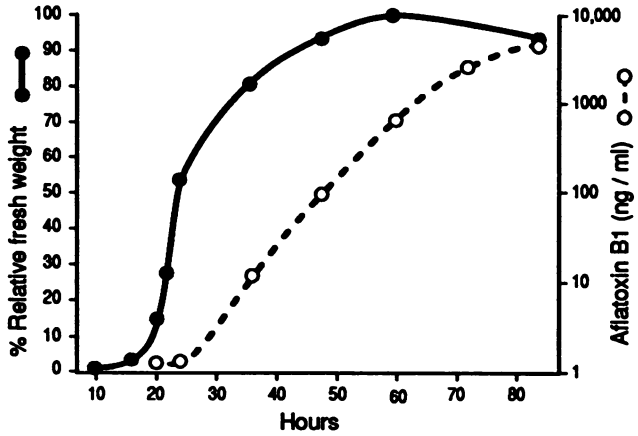

FIG. 2. Relationship of mycelium fresh weight and aflatoxin B1 production. Spores were directly inoculated into MMS plus 1% peptone medium at 10^6 spores per ml. Samples were harvested at the designated times. The culture medium was tested for aflatoxin production, and the mycelium was pressed dry, weighed, and used to isolate RNA for Northern hybridization. The relative fresh weight was based on the maximum fresh weight as 100%.

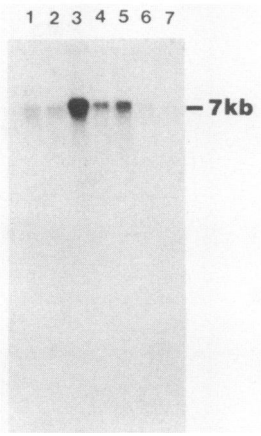

FIG. 3. Expression of clone 2-72 at different growth stages. Northern hybridization was carried out with clone 2-72 serving as a probe against poly(A)$^+$ RNAs isolated from different-aged mycelia. Lanes 1 to 7, RNA at 20, 22, 24, 36, 48, 60, and 84 h, respectively.

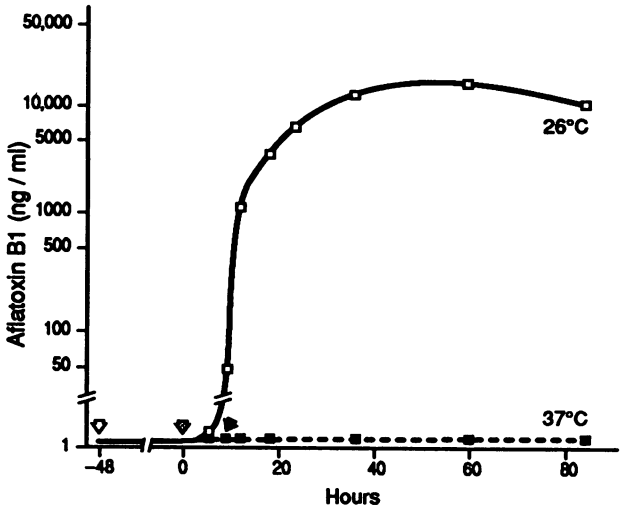

FIG. 4. Aflatoxin B1 production at different temperatures. Mycelia were shifted from PMS to MMS medium and cultured at the permissive (26°C) and nonpermissive (37°C) temperature, respectively. Aflatoxin B1 was detected in the MMS medium. Open arrow, inoculation time; stippled arrow, shifting time; solid arrow, time (9 h) when RNA was extracted.

weight accounted for less than 27% of the relative fresh weight. By contrast, the signal was strong in older cultures (24 h), when the fresh weight accounted for 54% of the relative fresh weight. Hybridization remained moderately strong during late log phase (36 and 48 h), weakened significantly at 60 h, and showed no signal among the poly(A)$^+$ RNAs isolated at 84 h. This hybridization pattern is similar to that found for the polyketide synthase gene in *Penicillium patulin* (4).

Hybridizations with three additional clones, designated 2-65, 2-75, and 1-36, also showed a similar pattern of developmental regulation (data not shown). The 3.4-kb band detected by clone 2-65, the 1.2-kb band detected by clone 2-75, and the 0.9-kb band detected by clone 1-36 showed results similar to those found for the 7-kb band recognized by clone 2-72. All of these hybridizations correlated positively with aflatoxin production.

There was a temporal relationship between poly(A)$^+$ RNA formation and aflatoxin production. The aflatoxin-associated probes hybridized with specific poly(A)$^+$ RNAs prior to and during aflatoxin formation. The lag period between the appearance of specific poly(A)$^+$ RNAs in the cell and aflatoxin in the medium can be accounted for by related cellular activities. These include RNA transcription, RNA processing, aflatoxin synthesis, and aflatoxin excretion from the mycelium into the culture medium. Reduction in the rate of aflatoxin biosynthesis after 84 h of growth was correlated with the absence of these poly(A)$^+$ RNAs, as measured with aflatoxin-related probes.

A different pattern of hybridization was found when clone 1-19 was used as a probe. The 1-19 probe hybridized strongly to two poly(A)$^+$ RNA bands (4.1 and 2.6 kb) at 60 and 84 h but showed weak hybridization at 24, 36, and 48 h (data not shown). This result contrasts with those obtained with probe 2-72 and the other probes (1-36, 2-65, and 2-75), which showed more intense hybridization with poly(A)$^+$ RNAs produced at 24, 36, and 48 h, weak hybridization at 60 h, and no hybridization at 84 h. Although all of these probes recognized poly(A)$^+$ RNAs associated with aflatoxin production, it is not clear at this time whether there is significance to the specificity of these hybridizations.

Expression of cloned sequences at different culture temperatures. The relation of aflatoxin production to mycelial growth at two different temperatures is shown in Fig. 4. Aflatoxin was not produced when cultures were incubated at a high temperature (37°C) but was strongly produced at a low temperature (26°C). Aflatoxin production was initiated 9 h after the transfer from PMS to MMS medium.

Poly(A)$^+$ RNAs were isolated from both high- and low-temperature cultures 9 h after the shift from PMS to MMS medium. In a Northern hybridization, clone 2-72 again hybridized with a 7-kb band. The hybridization signal was strong with the poly(A)$^+$ RNA isolated from 26°C cultures but very weak with poly(A)$^+$ RNA from 37°C cultures (Fig. 5). A similar result was obtained when clone 2-65 was used as a probe; it hybridized with a 3.4-kb poly(A)$^+$ RNA from 26°C cultures but not with poly(A)$^+$ RNA from 37°C cultures (data not shown). The absence of hybridization at 37°C can be explained by the lack of specific RNA rather than by a

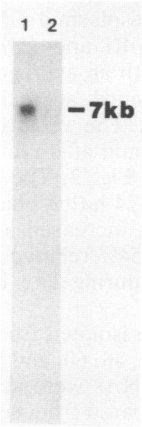

FIG. 5. Expression of clone 2-72 at different culture temperatures. Northern hybridization was carried out with clone 2-72 as a probe against poly(A)$^+$ RNAs isolated at different temperatures. Lane 1, RNA at 26°C; lane 2, RNA at 37°C.

general shortage of the total RNA. This is further suggested by the fact that the mycelium grew equally well at 37 and 26°C; moreover, there was no difference in the amounts of poly(A)$^+$ RNAs isolated at the two temperatures. These results further correlate these clones with aflatoxin biosynthesis.

DISCUSSION

Genes expressed under different growth conditions can be cloned either by differential screening or by subtraction hybridization, if regulation occurs at the transcription level and results in variable levels of extractable poly(A)$^+$ RNA (15). Such cloning in fungi has been achieved with galactose-inducible genes from *Saccharomyces cerevisiae* (27), with developmental genes from *Aspergillus nidulans* (30, 32), with dikaryon-expressed genes from *Schizophyllum commune* (13, 18, 22), and finally with the genes under control of the circadian clock from *Neurospora crassa* (16, 17).

A differential hybridization strategy was used in the present study to screen a genomic DNA library and to clone genes associated with aflatoxin biosynthesis in *A. parasiticus*. Three cDNA probes were used for screening, which represented different carbon sources in the media, i.e., PMS(−) versus MMS(+), and different growth stages, i.e., MMS(E) versus MMS(+). If induction of some genes were related to culture medium and growth stage, then screening with two factors should be more specific and effective than screening with a single factor. In other words, the clones which hybridized with probe MMS(+) but not with probe PMS(−) might still contain many genes specifically related to medium composition rather than aflatoxin biosynthesis. However, by comparing the hybridization results from probes MMS(E) and MMS(+), clones related only to medium composition could be eliminated. Recall that MMS(E) represented poly(A)$^+$ RNAs formed at early log phase, and MMS(+) represented poly(A)$^+$ RNAs formed during late log phase in the same medium.

The 19 clones associated with aflatoxin from the two-factor screening procedure may include regulatory and pathway genes. Some or all of the genes involved in the pathway could be controlled by coordinate regulation and expressed at the same time, as was found for the two enzymes which convert sterigmatocystin to aflatoxin B1 (12). The genes for β-lactam biosynthesis in *Penicillin* and *Aspergillus* spp. also seem to be controlled by a common mechanism, even though they are separately transcribed (26). Our hybridization experiments suggest that both medium and idiophase conditions could turn on many of the same genes. For example, from the 60 colonies that hybridized with probe MMS(+), 19 (about one-third) hybridized significantly more weakly or not at all with either the MMS(E) or the PMS(−) probe. Other experiments in progress have demonstrated that most of the 19 clones show no homology among themselves (data not shown).

Currently, two strategies are being used to clone the aflatoxin-related genes. One strategy is to purify pathway enzymes (7, 10) and use a synthesized oligonucleotide probe to identify genes in a cDNA library (6). This strategy may have limited application, since the enzymology and mechanistic details of many of the pathway steps are incompletely understood (14). These enzymes of secondary metabolism are also difficult to purify (12, 14, 19). The other strategy is complementation transformation, with a genomic DNA library used to complement aflatoxin-negative mutants and then rescue of the gene from the transformants or from the library (20, 25, 31). This strategy also faces difficulties because of the lack of an efficient transformation system in these aspergilli (25). The strategy we have used is a good alternative approach that adopts molecular techniques, makes use of existing data from other sources, and avoids some of the shortcomings of other strategies, such as difficulties associated with obtaining desired mutants, an inefficient transformation system, and the inability to do useful genetic analysis.

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