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REVIEW ARTICLE

Molecular biology of aflatoxin biosynthesis

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Aflatoxins: human and animal health; economic impact

Aflatoxins (*Aspergillus flavus* toxins) are biologically active secondary metabolites produced by certain strains of *Aspergillus parasiticus* and *A. flavus*. These ubiquitous fungi are capable of infecting a wide variety of crops, which, under certain conditions, can become contaminated with this potent mycotoxin. Ingestion of food or feed highly contaminated with aflatoxin can lead to acute toxicity including hepatotoxicity, teratogenicity, immunotoxicity, and even death (Dvorackova, 1990). Aflatoxin B1 (AFB1), the most abundant and toxic chemical form, is extremely mutagenic and is one of the most potent carcinogens ever tested in rats (Dvorackova, 1990; Eaton & Gallagher, 1994), suggesting that chronic exposure to very low levels of aflatoxin is cause for concern.

In humans, ingestion of aflatoxins is associated with hepatotoxicity (Dvorackova, 1990). Epidemiological studies also indicate that areas in the world with high levels of aflatoxin are correlated with high incidence of liver cancer. The presence of heptatitis B virus in these areas makes a direct cause-effect relationship difficult to establish. However, based on the available data, the International Agency for Research on Cancer designated AFB1 a probable human carcinogen. Because of this high level of concern about aflatoxin, the US Food and Drug Administration set action levels of 20 p.p.b. for food for human consumption (except milk, where the level is 0·5 p.p.b.) and 20–300 p.p.b. for most animal feeds (CAST, 1989). Other countries in the world have set even lower action levels.

From an economic standpoint, approximately 25 % of the world's crops are affected by mycotoxins annually (CAST, 1989). This equates to a direct cost of billions of dollars due to loss of crops and animals **plus** the hidden indirect costs incurred in monitoring the level of aflatoxins in crops and the decreased performance of farm animals that ingest aflatoxin and other mycotoxins. Elimination of aflatoxin is a critical economic and health problem in the US and throughout many other regions of the world.

As a testimony to their importance, aflatoxins have been the subject of numerous reviews in the past few years covering ecology (Cotty *et al.*, 1994), occurrence (Jelinek *et al.*, 1989), detection (Pestka, 1986, 1988), effects on human health (toxicity, carcinogenicity) (Bray & Ryan, 1991; Chang *et al.*, 1994; Chu, 1991; Dvorackova, 1990; Eaton & Gallagher, 1994), genetics (Bennett & Papa, 1988), biosynthesis (Bhatnagar *et al.*, 1992; Dutton, 1988) and compounds which affect biosynthesis (Zaika & Buchanan, 1987), and control of aflatoxin contamination (Park *et al.*, 1988; Park & Liang, 1993; Bhatnagar *et al.*, 1995); (for general reviews see CAST, 1989; Ellis *et al.*, 1991).

Because of the difficulty in effectively and economically controlling aflatoxin contamination of food and feed by traditional agricultural methods (see below), recent efforts in several laboratories have focused on developing an in-depth understanding of the molecular biology of the aflatoxin biosynthetic pathway. The goal of this review is to provide current information on the molecular biology of aflatoxin biosynthesis and how this information is being used to: (1) eliminate the toxin from the food chain; (2) understand the regulation and evolution of the aflatoxin pathway; and (3) understand the biological significance of aflatoxin to the producing fungus. This review is timely because it includes a discussion of several important breakthroughs which have resulted from intense research activity in the past 2 years – information which is not available in previously published reviews on the molecular biology of aflatoxin biosynthesis (Bhatnagar *et al.*, 1989; Keller *et al.*, 1992b; Linz & Pestka, 1992).

Since the aspergilli include opportunistic pathogens of mammals, insects and plants, furthering our understanding of the regulation of gene expression, development and secondary metabolism in this diverse genus may provide important clues into their ecology and biology, leading not only to effective control of aflatoxin but also to more general means for control of this entire group of pathogens.

Aflatoxin biosynthetic pathway

A. flavus, *A. nomius* and *A. parasiticus* are the only fungal species known to produce aflatoxins (Cotty *et al.*, 1994). However, as many as 20 different aspergilli, including *A. nidulans*, and species of *Bipolaris*, *Chaetomium*, *Farrowia* and

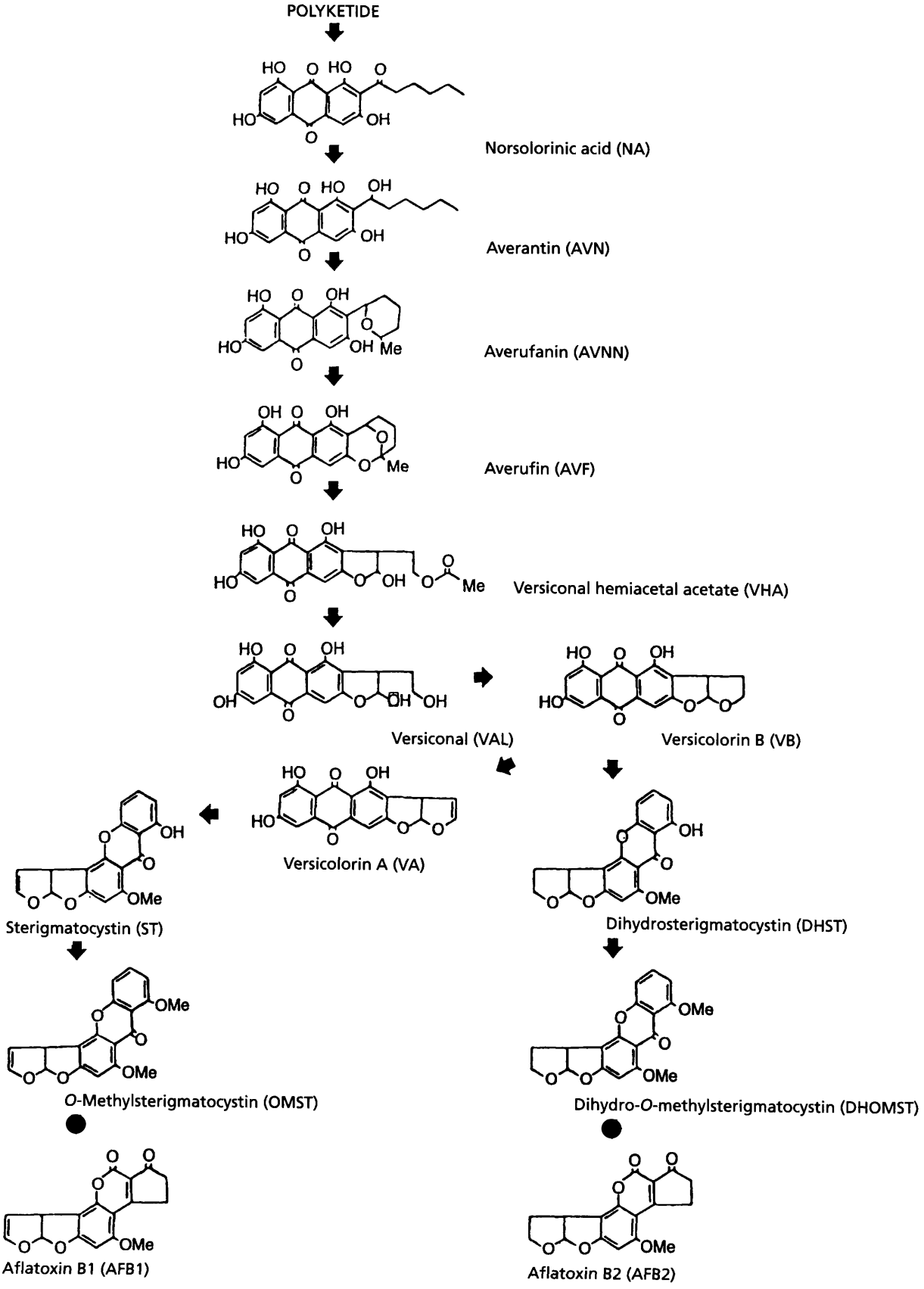

Fig. 1. Aflatoxin B1 and B2 biosynthetic pathway.

Monocillium, produce sterigmatocystin (ST) (Cole & Cox, 1981; Barnes *et al.*, 1994), a highly toxic intermediate in the AFB1 biosynthetic pathway. Even though the AFB1 biosynthetic pathway in *A. flavus* and *A. parasiticus* and the ST biosynthetic pathway in *A. nidulans* are believed to be similar, cooperative studies utilizing all three species are being pursued to identify any key differences which may exist in biosynthesis or regulation and to shed light on the evolution and acquisition of the pathway by the aspergilli and other genera.

Isolation and characterization of several mutants blocked in aflatoxin biosynthesis have been key contributions in elucidating the biochemistry and molecular biology of the aflatoxin pathway (reviewed by Bennett & Papa, 1988). Bioconversion experiments using these aflatoxin blocked mutants, metabolic inhibitors and stable isotope- or radio-isotope-labelled precursors or pathway intermediates have generated our current understanding of the order and mechanism of reactions in this complex biosynthetic pathway, which involves approximately 17 different enzymes (reviewed in Bhatnagar *et al.*, 1992; Dutton, 1988).

The initial step in generation of the polyketide backbone of AFB1 is proposed to involve polymerization of acetate and nine malonate units (with a loss of CO_2) by a polyketide synthetase (PKS) in a manner analogous to fatty acid biosynthesis (Bhatnagar *et al.*, 1992; Dutton, 1988). An alternative and perhaps more plausible hypothesis involves the synthesis of a 6-carbon hexanoate starter unit by a fatty-acid synthase (FAS), which is then extended by a PKS (without further ketoreduction) to generate a 20-carbon decaketide, noranthrone (Townsend *et al.*, 1991). In either scheme, noranthrone is then oxidized to the anthraquinone norsolorinic acid (NA) by a hypothesized oxidase. The rest of the proposed pathway is summarized in Fig. 1 (Yabe *et al.*, 1993; Bhatnagar *et al.*, 1992, 1995; Dutton, 1988). Versicolorin A (VA) is significant because it is the first molecule in the AFB1 pathway that contains a double bond at the 2,3 position in the difuran moiety. This double bond is the target for microsomal cytochrome P450 enzymes which generate a highly reactive epoxide resulting in activation and adduct formation with DNA and proteins (reviewed in Dvorackova, 1990). In contrast, aflatoxin B2 (AFB2), which lacks this double bond, is hundreds of times less carcinogenic (Dvorackova, 1990).

Several enzymes involved in the aflatoxin pathway are reported to have been purified to homogeneity. They include two separate *O*-methyltransferases (Bhatnagar *et al.*, 1988; Keller *et al.*, 1992c) which are involved in the conversion of ST to *O*-methylsterigmatocystin, an NA reductase (or possibly two separate enzymes) (Bhatnagar & Cleveland, 1990; Chuturgoon & Dutton, 1991) which is involved in the reversible conversion of NA to averantin (AVN), a cyclase, involved in the conversion of versiconal to versicolorin B (Lin & Anderson, 1992; Townsend *et al.*, 1991) and two versiconal hemiacetal acetate reductases (VHA reductase I and II; probably isozymes) which catalyse the reaction from versiconal hemiacetal acetate to versiconol acetate (Matsushima *et al.*, 1994). These purified enzymes provided important tools for cloning of genes.

Gene cloning strategies/structure and function of cloned genes

Cloning of genes involved in aflatoxin biosynthesis is the key to understanding the molecular biology of the pathway. Cloned genes are useful probes for elucidating the molecular mechanisms that regulate the timing and level of expression of these genes. Two different strategies have been successfully utilized in the cloning of aflatoxin biosynthetic genes. A genetic complementation approach was successful in the isolation of genes encoding three enzymes in the pathway, *nor-1, ver-1* and *uvm8*, and one regulatory gene, *aflR*. For introduction of DNA into the fungus, transformation systems were developed for *A. parasiticus* (Skory *et al.*, 1990; Horng *et al.*, 1990) and *A. flavus* (Woloshuk *et al.*, 1989).

The *nor-1* (originally *nar-1* for NA related; Chang *et al.*, 1992) and *ver-1* genes (Skory *et al.*, 1992) were cloned by complementation of aflatoxin blocked mutants B62 (an *niaD* mutant derived from *A. parasiticus* ATCC 24690, *nor-1, brn-1*, Lee *et al.*, 1970) and CS10 (an *niaD* mutant derived from *A. parasiticus* ATCC 36537, *ver-1, wh-1*, Bennett & Goldblatt, 1973), which accumulate the brightly coloured pathway intermediates NA (brick-red) and VA (yellow), respectively. Complementation was achieved by introduction of a cosmid DNA library generated using genomic DNA from a wild-type aflatoxin-producing strain of *A. parasiticus* (SU-1). The functionally homologous *verA* gene of *A. nidulans* was isolated by hybridization of *ver-1* to an *A. nidulans* genomic DNA library (Keller *et al.*, 1994). The predicted amino acid sequences of the *ver-1* and *verA* gene products are nearly identical (Keller *et al.*, 1994), illustrating the high degree of identity between aflatoxin biosynthetic genes among these *Aspergillus* species. The predicted amino acid sequences of *nor-1, ver-1* and *verA* contain an NAD(P)H binding motif near the amino terminus and show significant identity (33% for *ver-1*/*verA*; 23% for *nor-1*) to several NADPH- and NADH-dependent reductase/dehydrogenase enzymes. Each sequence also contains a short-chain alcohol dehydrogenase motif (Trail *et al.*, 1994a).

To confirm the role of these genes in aflatoxin biosynthesis, recombinational inactivation (gene disruption) was conducted in toxigenic strains of *A. parasiticus* (*nor-1*, Trail *et al.*, 1994a; *ver-1*, Liang & Linz, 1994) and *A. nidulans* (*verA*, Keller *et al.*, 1994). Disruption of the *verA* gene resulted in loss of detectable ST and accumulation of VA by *A. nidulans*, confirming its role in conversion of VA to ST. Similarly, disruption of *ver-1* blocked the aflatoxin pathway, resulting in VA accumulation. Disruption of *nor-1* resulted in accumulation of large quantities of NA. Disrupted strains retained their ability to produce low levels of aflatoxin, supporting the hypothesis that there is one or more alternative routes (or enzymic activities) in the aflatoxin pathway to synthesize averufin from NA (Yabe *et al.*, 1993; reviewed in Bhatnagar *et al.*,

1992). Recently, a *nor-1*/maltose-binding protein (MBP) fusion protein was expressed in *E. coli* (Zhou & Linz, 1994). Crude *E. coli* cell extracts containing the fusion protein converted NA to AVN, but only in the presence of NADPH, confirming the prediction that *nor-1* encodes a reductase which converts NA to AVN.

The gene *uvm8* was cloned by complementation of an aflatoxin blocked mutant, *uvm8*, derived by UV mutagenesis of *A. parasiticus* mutant strain B62 (*niaD, brn-1, nor-1*; Mahanti *et al.*, 1994). Metabolite conversion studies confirmed that *uvm8* has two blocks in the AFB1 pathway, one block at *nor-1* and the other one prior to *nor-1*. The predicted peptide sequence of extensive regions of the *uvm8* gene product showed a high degree of similarity (67%) and identity (48%) to the β-subunit of FASs (FAS1) from *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Kottig *et al.*, 1991). *uvm8* was therefore hypothesized to encode an FAS activity necessary for synthesis of the proposed hexanoate starter. Since two keto groups in hexanoate are completely reduced to hydrocarbon, an FAS involved in its synthesis would be expected to contain three key enzyme activities, ketoreductase, dehydratase and enoyl reductase, in addition to a β-ketoacyl-synthase. Limited nucleotide sequencing identified an enoyl reductase domain in *uvm8* (based on identity with *S. cerevisiae* FAS1) which would not be theoretically necessary for the aflatoxin PKS. The *uvm8* gene was disrupted in *A. parasiticus* and the disrupted strains did not accumulate any detectable AFB1 or pathway intermediates consistent with a functional role in polyketide backbone synthesis.

A second approach for isolation of genes, reverse genetics, relied on the purified pathway enzymes discussed above. Where purification has been possible, production of antibodies to the enzyme, and isolation of the gene from a cDNA expression library in *E. coli*, can be accomplished. This procedure was utilized to clone the *omt-1* gene from *A. flavus* encoding the *O*-methyltransferase activity responsible for conversion of ST to *O*-methylsterigmatocystin (Yu *et al.*, 1993; see Fig. 1). An *A. parasiticus* cDNA library, generated from RNA from an aflatoxin induced culture, was screened with antibodies raised against the native methyltransferase. The predicted amino acid sequence derived from the cloned cDNA contained a motif found in other *S*-adenosylmethionine-dependent methyltransferases. The purified native protein and a fusion protein expressed from the cDNA in *E. coli* both demonstrated substrate-specific methyltransferase activity. To date, *omt-1* is the only pathway gene cloned by the reverse genetics approach. However, this approach should be successful in cloning several other genes encoding the purified pathway enzymes.

Another molecular genetic approach for gene cloning, subtractive hybridization, has been used by Feng *et al.* (1992) to isolate several genes whose pattern of expression coincides with aflatoxin production in *A. parasiticus*. This method is not based on specific knowledge of the function of the gene product, as in the two previous methods, and can therefore be advantageous when the timing of induction of gene expression is known but pure enzymes or blocked pathway mutants are not available. To date, the specific identification of the activities of genes isolated by this method has not been reported.

Regulation of aflatoxin gene expression

Polyketides are a large and diverse family of secondary metabolites which are produced primarily by actinomycetes, fungi and higher plants, but are also synthesized in other organisms including animals (reviewed in Hopwood & Khosla, 1992). Regulation of synthesis of these secondary metabolites is distinct from regulation of primary metabolism, although secondary metabolism relies on primary metabolism for energy, enzyme cofactors and building blocks (i.e. acetate). The effect of primary metabolism on the biosynthesis of aflatoxin has been reviewed by Luchese & Harrigan (1993).

In culture, *A. parasiticus* and *A. flavus* produce aflatoxins during idiophase, when exponential growth has slowed or ceased and secondary metabolites are formed. Using transcription and translation inhibitors, Buchanan *et al.* (1987) demonstrated that *de novo* protein synthesis is required for aflatoxin production. Other studies demonstrated that the activities of at least four enzymes involved in the pathway are not detected until idiophase (Anderson & Green, 1994; Chuturgoon *et al.*, 1990; Cleveland & Bhatnagar, 1990). During batch fermentation of *A. parasiticus*, the *ver-1* and *nor-1* RNA transcripts accumulated most rapidly during the transition between active growth and stationary phase (Skory *et al.*, 1993). The accumulation of the RNA transcripts from the *aflR* gene, proposed to encode a key regulatory protein (see later), was shown to follow a similar pattern (Payne *et al.*, 1993). The coordinate transcription of these genes suggested that they are regulated in part at the level of transcription, perhaps by a common regulatory factor.

A gene proposed to encode one important regulatory factor, the *afl-2* gene, was cloned by complementation of an aflatoxin-nonproducing mutant using a wild-type genomic DNA library from *A. flavus* (Payne *et al.*, 1993). Genetic evidence as well as metabolite feeding studies suggested that *afl-2* is involved in aflatoxin biosynthesis before NA. For example, a mutant strain of *A. flavus* blocked at *afl-2* was unable to convert a number of exogenously supplied pathway intermediates to aflatoxin, indicating that key pathway enzymes were not present. Complementation of mutant strains with the wild-type *afl-2* gene simultaneously restored expression of several aflatoxin pathway enzyme activities in crude cell extracts, a characteristic expected of a gene encoding a *trans*-acting regulatory factor.

In *A. parasiticus*, *apa-2* was cloned on the basis of overproduction of aflatoxin pathway intermediates after transformation with a single cosmid clone (NorA) that contained both aflatoxin genes *nor-1* and *ver-1* (Chang *et al.*, 1993). *apa-2* complemented an *A. flavus afl-2* mutant strain to aflatoxin production suggesting that *apa-2* and *afl-2* are functional homologues. Nucleotide sequence analysis confirmed the genetic data – these genes share greater than 95% nucleotide sequence identity (Chang *et al.*, 1993). A cysteine-rich zinc cluster motif, Cys-Xaa2-

Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys, was observed in the predicted amino acid sequences of *apa-2* and *afl-2* (Chang *et al.*, 1993; Woloshuk *et al.*, 1994). This zinc cluster motif is characteristic of a group of fungal transcriptional activators, the best studied of which is GAL4 of *S. cerevisiae*. GAL4 regulates the transcription of genes involved in yeast galactose utilization. Because the preponderance of data strongly suggested that *apa-2* and *afl-2* are positive regulators of aflatoxin synthesis, these homologues have been renamed *afl*R (Payne *et al.*, 1993).

The aflatoxin gene cluster

Because *A. parasiticus* and *A. flavus* do not have a known perfect (sexual) stage, classical genetic studies have been conducted using the parasexual cycle. Parasexual analysis of eight aflatoxin blocked mutants in *A. flavus* suggested that they were all genetically linked to markers on linkage group VII (Papa, 1984). However, attempts to demonstrate linkage of *nor-1* and *ver-1* by parasexual analyses have been confusing because of problems inherent in analysing ploidy levels of segregants and the nonrandom segregation of certain genes during haploidization (reviewed by Bennett & Papa, 1988).

Molecular genetic analyses have now provided proof that many of the genes involved in aflatoxin biosynthesis in *A. parasiticus* and *A. flavus* are physically clustered on one chromosome. During the cloning and characterization of the *nor-1* and *ver-1* genes from *A. parasiticus*, one cosmid, NorA, was identified that hybridized to probes of both genes. This tentative evidence for linkage was later confirmed by physical mapping of the corresponding region in the fungal genome in *A. parasiticus* (Trail *et al.*, 1995). *afl*R, *uvm8* and *omt-1* were later mapped to this cluster and to a similar cluster of aflatoxin genes in *A. flavus* (Bhatnagar *et al.*, 1994; Trail *et al.*, 1995). One intriguing observation is that the physical order of genes in the cluster appears to be similar to the order of enzyme reactions catalysed by their gene products. Whether there is any functional significance to this feature is not clear.

Since as many as 17 enzyme activities are thought to be required to complete aflatoxin synthesis it was hypothesized that the cosmid NorA (and the corresponding region in *A. flavus*) encoded several other pathway enzymes. To determine the size, location and pattern of expression of other genes in the cluster, a transcriptional map of the genomic DNA insert in cosmid NorA was completed (Trail *et al.*, 1995). Twelve unique RNA transcripts were localized to this cluster. Because the timing of their expression was similar to that observed for *nor-1* and *ver-1*, they were tentatively identified as aflatoxin genes.

Genetic disruption of a gene (encoding a 7·0 kb transcript) located adjacent to *nor-1* in the gene cluster blocked VA production in a VA-accumulating mutant, CS10 (Trail *et al.*, 1995), and OMST production in an OMST-accumulating strain (P.-K. Chang, J. W. Cary, J. Yu, D. Bhatnagar & T. Cleveland, personal communication). Predicted amino acid sequence data from an extensive region of this gene showed a high degree of identity to the β-ketoacyl-synthase (67 % identity) and the acyltransferase (32 % identity) functional domains (Trail *et al.*, 1995) of the *wA* gene product in *A. nidulans* which encodes a PKS involved in conidial pigment production (Mayorga & Timberlake, 1992). P.-K. Chang and others (personal communication) also report high homology to the acyl carrier protein domain of the *wA* gene product. It is possible that this putative aflatoxin PKS is involved in extending the hexanoate starter unit synthesized by *uvm8*.

The specific role of other genes in the cluster localized by transcript mapping can be similarly identified using a nucleotide sequence approach combined with biochemical analyses of genetically disrupted strains. 'Feeding' disrupted strains with aflatoxin pathway intermediates and examining their ability to convert these substrates to the subsequent intermediates can help to identify the step at which the gene disruption occurs. This approach to identifying gene function is being applied to another interesting gene located adjacent to *nor-1* (encodes a 6·5 kb transcript). Nucleotide sequence analysis of a limited portion of this gene revealed that the predicted protein shares a high degree of identity (51 % over 150 amino acid residues) to the enoyl-reductase domain in the same FAS1 products from yeast as were observed in analysis of *uvm8* (Trail *et al.*, 1995). It is possible that synthesis of the hexanoate starter requires two FAS subunits (α and β; encoded by unique genes) analogous to those of yeast. Gene disruption combined with feeding studies will allow this hypothesis to be tested.

Clustering of genes involved in secondary metabolism is a common phenomenon. For example, different species of *Streptomyces* produce a variety of polyketide-derived antibiotics, including erythromycin, tetracenomycin, actinorhodin, griseusin and granaticin (reviewed in Hopwood & Khosla, 1992; Martin & Liras, 1989). Several genes contained in their biosynthetic pathways show a high degree of identity with genes in analogous pathways and are clustered in similar patterns on the chromosome.

The clustering of fungal genes involved in synthesis of secondary metabolites has also been reported. The genes encoding enzymes in penicillin and cephalosporin (members of β-lactam class of antibiotics) pathways of *Penicillium chrysogenum* and *Cephalosporium acremonium* (reviewed in Aharonowitz & Cohen, 1992), *A. nidulans* (Montenegro *et al.*, 1992), as well as the genes in the trichothecene pathway (toxic sesquiterpenes) in *Fusarium sporotrichioides* (Hohn *et al.*, 1993), occur as gene clusters. Recent findings, however, suggest that the clustering of fungal genes is not limited to synthesis of secondary metabolites. In the filamentous fungus *Alternaria alternata*, some of the genes involved in melanin biosynthesis (a dark-brown polyketide-derived pigment) are clustered within a 30 kb stretch of genomic DNA (Kimura & Tsuge, 1993).

The significance (if any) of gene clustering in the function, regulation or evolution of the aflatoxin biosynthetic pathway has not yet been elucidated. However, with increasing evidence that chromatin structure is involved in gene regulation (Cavalli & Thoma, 1993; Gross *et al.*,

1993; Wolffe, 1994), a role for chromosome structure in cluster expression is possible. This is an area that has not been examined and one to which the techniques of molecular biology can be applied.

Duplication of aflatoxin genes

In physical mapping studies of the cosmid NorA, it became apparent that there are at least two copies of the *ver-1* gene, *ver-1A* and *ver-1B*, located in separate regions in the *A. parasiticus* genome (Liang & Linz, 1994). By comparing the restriction enzyme polymorphisms present in these two chromosomal copies with the cloned *ver-1* gene, it was confirmed that the gene cloned originally was *ver-1A*. *ver-1B* was subsequently cloned and its nucleotide sequence determined. These genes were found to share 93% nucleotide sequence identity. A stop codon was identified near the middle of the predicted *ver-1B* gene transcript suggesting that it may encode a truncated polypeptide that has little or no function. A duplicated chromosomal region extending approximately 12 kb upstream from *ver-1A* and *ver-1B* was identified which also contains an additional copy of *afl*R (Liang & Linz, 1994). Duplication of *ver-1* and *afl*R genes in *A. parasiticus* may explain the higher stability of toxin production in *A. parasiticus* as compared to *A. flavus*, in which such a duplication is not apparent. More than 90% of *A. parasiticus* isolates produce aflatoxin whereas 50% (or less) of *A. flavus* isolates are toxigenic (Bennett & Papa, 1988).

Chromosomal organization of aflatoxin genes

Keller *et al.* (1992a) successfully used pulsed field gel electrophoresis as a tool for genetic analyses of the aflatoxigenic fungi. Genetic karyotyping and Southern blot analyses with several different gene probes demonstrated the similarities between the *A. flavus* and *A. parasiticus* genomes and dissimilarities to those of *A. nidulans* and *A. niger*. Under identical electrophoretic conditions, *A. flavus* (five to eight visible chromosomal bands), *A. parasiticus* (five to six chromosomal bands) and *A. versicolor*, a related species which has been reported to produce precursors in the aflatoxin pathway (six chromosomal bands), showed similar but variable numbers of chromosomes. The total genome sizes of these fungi were similar to the size reported for *A. nidulans* and *A. niger* (31–38·5 Mb). An additional and potentially important observation resulted from these studies. The karyotype patterns in 19 different *A. flavus* isolates were unique and it has been suggested that this sort of genetic variability is very common in this species (Bennett & Papa, 1988). The size variation could be an indication of chromosomal rearrangement via gross translocation leading to unique karyotype patterns. Imperfect fungi may be tolerant of such rearrangements because asexual reproduction (via mitosis) only requires segregation of identical chromatids, unlike sexual reproduction, which requires pairing of identical chromosomes and which is under a strict genetic control. Of practical significance, the variability in genomes of different isolates of *A. flavus* may be related to the apparent instability in the ability to produce aflatoxins.

In a related study, Keller *et al.* (1994) recently showed that the *verA* gene of *A. nidulans* hybridizes strongly to chromosome IV (2·9 Mb in size). Using similar procedures it should now be possible to determine the locations of duplicated regions of the aflatoxin gene clusters on the same or different chromosomes in *A. parasiticus*.

Ongoing research

Current research on the molecular biology of aflatoxin synthesis is focused on two main areas: (1) the structure, function, organization and comparative mapping of the aflatoxin (or ST) genes and gene clusters in *A. parasiticus*, *A. flavus* and *A. nidulans*; and (2) identification of molecular mechanisms which regulate pathway genes (regulatory genes; aflatoxin promoter structure and function).

Gene/cluster structure and function

Nucleotide sequence analysis and transcript mapping of the majority of the genes in the clusters in *A. parasiticus* and *A. nidulans* should be completed in the near future while disruption studies are continuing as candidate genes are identified. In related work, the activity and localization of pathway enzymes is being pursued. For example, *nor-1* and *ver-1* maltose-binding protein fusion products have been expressed in *E. coli* and polyclonal antibodies (pAb) have been generated which appear to recognize the native fungal proteins (Liang & Linz, 1994; Zhou & Linz, 1994). These antibodies, along with available antibodies to the *omt-1* protein, will be used to localize these proteins in the cell and to determine if proteins act independently or in enzyme complexes. Preliminary data using the *ver-1* polyclonal antibody suggest that *ver-1* proteins are mainly localized in the membrane fraction of the fungal cell (Liang & Linz, 1994). Immunolabelling will also be useful in exploring the kinetics and level of expression of aflatoxin enzymes in host plant tissues for studies on plant resistance mechanisms against aflatoxin production.

Molecular mechanisms which regulate pathway genes

A second putative regulatory locus (besides *afl*R), *afl-1*, was identified by Leaich & Papa (1974) in *A. flavus* using UV mutagenesis and was later determined to be linked to *nor-1* by parasexual analysis (reviewed by Bennett & Papa, 1988). *afl-1* mutants are functionally dominant in diploids resulting in loss of aflatoxin production. Recent studies (Woloshuk & Yousibova, 1994) determined that the *afl-1* mutation results in suppression of transcription of the three structural genes tested (*nor-1, ver-1* and *omt-1*). Transcription of *afl*R was normal in these strains. Future studies will focus on cloning *afl-1* and determining its role in regulation.

With the cloning of *afl*R and the identification of *afl-1*, study of the mechanisms of control exerted by the products of these genes is the next logical step. The current approach for conducting these studies is to identify the *cis*-acting sites and *trans*-acting proteins that

regulate aflatoxin gene function. The *nor-1, ver-1* and *aflR* promoters have been fused to the *E. coli* gene encoding β-glucuronidase (*uidA*), the GUS gene, whose gene product can be easily detected with colorimetric or fluorometric assays (Trail *et al.*, 1994b; Wu & Linz, 1994). Fungal strains containing these reporter constructs are now being used: (1) to detect the induction of aflatoxin genes in the fungus grown under various culture conditions and to trace the fungus in the plant under various conditions; and (2) to identify the *cis* regulatory regions important in control of these promoters through deletion or site-directed mutation analyses.

Promoter regions are also being analysed by mobility shift assays. Proteins which specifically bind can then be identified and purified. Preliminary data suggest that there are at least two specific DNA/protein interactions in the *nor-1* promoter (Trail *et al.*, 1994b). The functional significance of these interactions remains to be demonstrated.

Applications of molecular biology to aflatoxin elimination, evolution and biological significance of the aflatoxin pathway

The study of aflatoxin biosynthesis in culture provides a model system for understanding the biosynthesis of aflatoxin on natural substrates. However, factors important to the regulation of aflatoxin biosynthesis in the host plant may be different from those functioning in culture. Future work must involve more studies of the fungus in the host plant. These studies in turn may lead to new techniques for toxin control and an increased understanding of the evolution and biological function of the aflatoxin pathway.

Elimination of aflatoxins from food and feed

Fig. 2 illustrates how the contributions of molecular biology described above can be applied to the elimination of aflatoxin from food and feed. Each of these applications will be summarized briefly.

Several approaches (grouped into preharvest and postharvest strategies) are in current use or have been proposed for use in reducing or eliminating aflatoxin from the food chain. Preharvest strategies are designed to block fungal infection of the host plant (crop) or to block the ability of the fungal pathogen to grow or synthesize aflatoxins on the plant and, in the future, are likely to have the greatest impact on human and animal health. Costly and/or ineffective postharvest elimination strategies involving aflatoxin screening/detection, removal/adsorption, decontamination or altered aflatoxin metabolism/DNA adduct formation will not need to be relied on as critical treatment steps but will provide a safety net to remove low levels of aflatoxins that may escape preharvest control.

Current preharvest approaches including irrigation, application of fungicides or insecticides and use of resistant

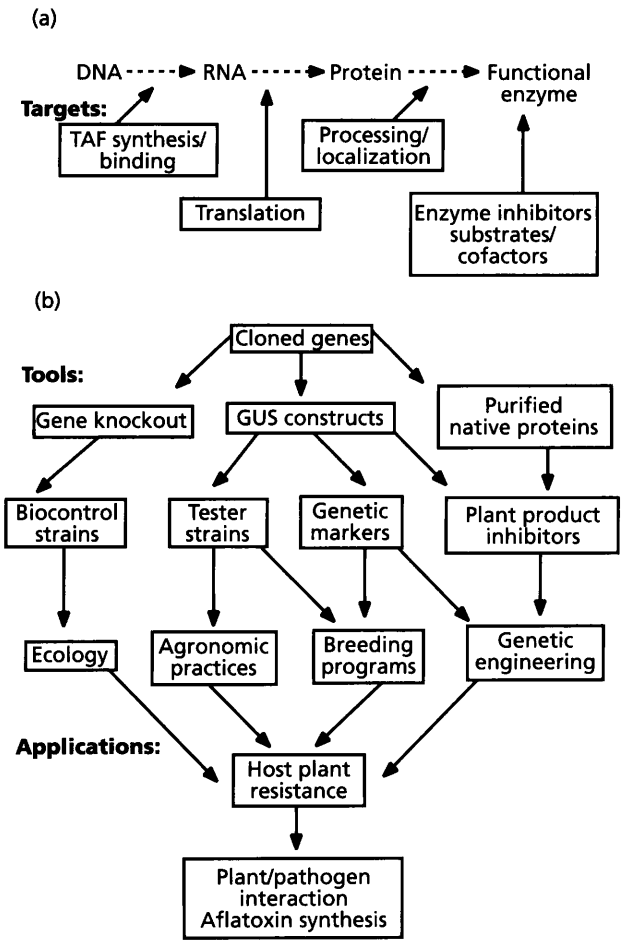

Fig. 2. Applications of molecular biology to aflatoxin elimination. (a) Targets for inhibition of expression of aflatoxin genes. Each step in gene expression, transcription, RNA transport and processing, translation, protein processing and localization can in theory be inhibited by natural plant products or other agents which can be identified using GUS reporter constructs or polyclonal antibodies for each gene described in the text. TAF, *trans*-acting factor (regulatory protein). (b) Tools derived from cloned aflatoxin genes and their potential application to increase host plant resistance to fungal growth, infection or toxin biosynthesis. The primary tools under development are biocontrol strains, fungal strains containing GUS reporter constructs (i.e. tester strains) and polyclonal antibodies raised to pure native proteins or proteins expressed in *E. coli*.

or regionally adapted crop varieties fall short of effective control. Use of chemicals or irrigation is often environmentally unacceptable or too costly while genetically stable highly resistant crops have not been successfully obtained using conventional breeding methods. However, several promising preharvest strategies for aflatoxin control have been proposed for future use. These strategies are focused on two main areas: (1) genetically engineered crops to reduce fungal growth or inhibit aflatoxin biosynthesis (long-term approach); and (2) utilization of biological control organisms to competitively exclude the toxigenic fungus from infecting the crop (short-term approach). These and other potential

applications of molecular biology to aflatoxin elimination have been recently reviewed in detail (Bhatnagar *et al.*, 1995) but are summarized briefly here to provide a framework for the discussion of molecular biology of aflatoxin synthesis.

Genetic engineering of crops. This approach utilizes molecular genetics to enhance expression of genes controlling natural (endogenous) resistance and/or to introduce resistance genes from other sources into susceptible plants. *A. parasiticus* and *A. flavus* are weak pathogens of the reproductive organs of the plant and are particularly aggressive in mature seeds where high concentrations of oil are present (Cotty *et al.*, 1994). Identification of the signals exchanged between host and pathogen which stimulate aflatoxin production in susceptible plants under host stress or which inhibit toxin formation in 'naturally resistant' crops should aid in successful genetic manipulation of crops. Much work still needs to be done in this area; however, the approach holds promise because it may be relatively straightforward to enhance natural or endogenous resistance by modulation of expression of genes which are a normal part of the plant genome.

Resistance genes from other sources in theory may be obtained through identification of naturally occurring plant compounds that inhibit growth and/or aflatoxin production by *A. flavus* and *A. parasiticus*. Crude botanical extracts that exhibit these properties have been identified (reviewed by Zaika & Buchanan, 1987). Genes encoding the synthesis of these novel compounds can be introduced into crops by genetic engineering. Clearly, success will be more easily obtained if one or two genes allow the biosynthesis of the compound and will only be obtained if the compound is nontoxic to humans, animals and the engineered plant. In addition, the additional genes must be expressed in the engineered plant in the right organ at the right time. The aflatoxin gene/GUS reporter constructs are extremely valuable tools for identifying plant compounds (or other agents) which stimulate or inhibit fungal infection, growth or toxin biosynthesis.

Biological control. Strains of *A. flavus* and *A. parasiticus* have shown promise in reducing the level of the resident fungal population and have demonstrated a significant reduction (80–90%) in aflatoxin contamination in greenhouse and field studies (Dorner *et al.*, 1992; Cotty & Bhatnagar, 1994; reviewed in Cotty *et al.*, 1994). Because this approach depends on survival and successful occupation of an ecological niche by the biocontrol strain, identification of the environmental factors that favour certain isolates of *A. flavus* and *A. parasiticus* over others must be understood. One interesting feature of this approach, which must be considered for its successful implementation, is that strains of *A. flavus* seem to replace other strains of *A. flavus* more effectively than *A. parasiticus* and vice versa (Horn *et al.*, 1994). Therefore, it is likely that combinations of strains of both species will be required.

Recent studies have suggested that naturally occurring nontoxigenic isolates of *A. flavus* may have the genetic capability to synthesize AFB1 (Rarick *et al.*, 1994) under as yet undetermined environmental conditions. With the use of a molecular genetics approach, genetically stable atoxigenic biocontrol strains of *Aspergillus* that are known to compete well can be generated by specific deletion of key genes in the biosynthetic pathway once these genes have been identified. Using this gene disruption technology, at least one genetically engineered fungal biocontrol strain (*uvm8* disruption strain – Dis3) has been made available for field testing (Mahanti *et al.*, 1994).

Evolution

There is a high degree of sequence identity between aflatoxin genes (*ver-1, aflR, omt-1*) in *A. parasiticus*, *A. nidulans* and *A. flavus*. The organization of the gene cluster also is well conserved. Interestingly, the *nor-1* and *ver-1* genes are present in *A. sojae*, *A. oryzae* and nontoxigenic *A. flavus* strains (Rarick *et al.*, 1994). These data suggest that the progenitor *Aspergillus* strain that gave rise to the current species under study also contained the AFB1 or ST pathway (it will be interesting to determine if *A. nidulans* has the genetic capacity to produce AFB1; i.e. genes for the *O*-methyltransferase and oxidoreductase required to convert ST to AFB1). Physical clustering may also suggest that the progenitor strain obtained the pathway intact via horizontal transfer from some other organism (i.e. *Streptomyces* spp. produce anthraquinone polyketide antibiotics, structurally related to intermediates in AFB1 synthesis). Alternatively conservation of cluster organization may suggest that function or regulation of aflatoxin synthesis relies on an intact structural organization. One other possibility which should receive further study is that the aflatoxin pathway evolved from a pre-existing pathway for synthesis of a fungal polyketide, perhaps a mycelial or spore pigment. In support of this notion, the putative aflatoxin PKS shows a high degree of sequence identity to the PKS involved in conidial pigment synthesis in *A. nidulans*. Interestingly, the chemical structure of an intermediate in conidial pigment synthesis in *A. parasiticus* (naphthapyranone, a polyketide) also bears strong resemblance to NA (Brown *et al.*, 1993). A similar study determined that an ascospore pigment (ascoquinone A) in *A. nidulans* is a dimer of an anthraquinone and is likely to be polyketide in origin (Brown & Salvo, 1994). Additional data which may support a link between pigment synthesis and aflatoxin synthesis were recently provided in studies on melanin biosynthesis in *Magnaporthe grisea* (Vidal-Cros *et al.*, 1994). The predicted amino acid sequence of the gene encoding a polyhydroxynaphthalene reductase involved in melanin biosynthesis was reported to share 56% identity to the *ver-1* gene product in *A. parasiticus*. This might suggest that these biosynthetic pathways (or parts of the pathways) are derived from a common ancestral polyketide pathway.

Biological significance of aflatoxins: a role in fungal development?

The size of the aflatoxin cluster and the striking conservation of genes and cluster organization strongly suggest that aflatoxins play a key role in the life cycle or

survival of the fungus. Are there any clues as to what this function might be? Conidia (asexual spores of the aspergilli) and sclerotia (resting/survival structures) are two major sources of inoculum for survival or spread of these filamentous fungi. The interrelationship between sclerotia production and aflatoxin (if any) is not clear. A study by Bennett & Horowitz (1979) suggested that there is no correlation between sclerotia production and aflatoxin in toxigenic and atoxigenic strains of *A. flavus*. In contrast, other studies suggested that the regulation of aflatoxin synthesis in toxigenic strains does influence sclerotia development (reviewed in Cotty *et al.*, 1994). Preliminary studies using the tools of molecular biology have shown that mutations (UV or gene disruption) which result in accumulation of certain aflatoxin pathway intermediates (i.e. AVF, VA) also result in inhibition of development of sclerotia. Genetic blocks which eliminate AFB1 and intermediate synthesis (i.e. *uvm8*, 7·0 kb) result in enhanced sclerotia production (Skory *et al.*, 1992; Trail *et al.*, 1995). Restoration of function by complementation also restores normal sclerotia development. These results suggest that aflatoxin synthesis and fungal development may be connected. A continuation of these studies may uncover the nature and significance of such a link.

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