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Isolation and Sequence Analysis of a β-Tubulin Gene from *Aspergillus flavus* and Its Use as a Selectable Marker

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An altered β-tubulin gene that confers resistance to benomyl [whose active ingredient is 2-(methoxycarbonylamino)benzimidazole (MBC)] was isolated from a DNA library of *Aspergillus flavus* and used as a selectable marker for transformation. The β-tubulin gene was cloned into a plasmid vector containing the *pyr-4* gene of *Neurospora crassa*, and transformants were selected either for uracil prototrophy or MBC resistance. Transformants selected for uracil prototrophy were of three phenotypic classes: sensitive, intermediate, and resistant to MBC. Transforming DNA appeared to integrate at several sites in the genome, with the more resistant phenotypes having more copies of the altered β-tubulin gene than the sensitive and intermediate phenotypes. Transformants were also selected on medium containing MBC. The average frequency of transformation (1 to 3 transformants per μg of transforming DNA) was lower than that obtained by selection for uracil prototrophy, presumably because of failure to select transformants that contained few copies of the altered β-tubulin gene. The sequence of the β-tubulin gene was determined and compared with the published sequence of the *benA* gene of *A. nidulans*; the β-tubulin gene was found to be highly conserved between the two *Aspergillus* species. Notable differences were that the β-tubulin gene of *A. flavus* lacks intron 6 present in *benA* and has an additional leucine at position 148. This is the first gene sequence reported from an aflatoxin-producing fungus and adds to the growing body of knowledge of the β-tubulin genes and their use as selectable markers for transformation of filamentous fungi.

The filamentous fungus *Aspergillus flavus* produces a family of toxic and carcinogenic secondary metabolites called aflatoxins in a number of important food sources, including corn and peanuts. Extensive studies on the biosynthesis of aflatoxin have resulted in the isolation of a number of pathway intermediates and a proposed pathway (6, 26), yet only two of the enzymes involved in the pathway have been isolated and characterized (4, 11) and little is known about the regulation of aflatoxin biosynthesis.

Because enzymes in the pathway are difficult to isolate by biochemical methods, we are attempting to isolate genes coding for pathway enzymes via complementation of mutants blocked in aflatoxin biosynthesis. We recently developed a genetic transformation system for *A. flavus* by using the *pyr-4* gene from *Neurospora crassa* as a selectable marker (23). This system allows easy selection of transformants, but it requires that recipient strains be uracil auxotrophs. In studies in which several pathway enzyme mutants are to be transformed, it is desirable to have a dominant selectable marker that allows selection without prior manipulation of the recipient strain.

The fungicide benomyl has been used as a dominant selective marker in other fungi (18); however, our attempts to select transformants by using heterologous β-tubulin genes from *A. nidulans* and *N. crassa*, known to confer benomyl resistance, were unsuccessful (C. P. Woloshuk and G. A. Payne, unpublished data). Because homologous genes have increased transformation efficiencies in some systems (18, 22), this study was initiated to isolate a native β-tubulin gene from a benomyl-resistant strain of *A. flavus* and use it as a selectable marker for transformation. In this communication we also report the nucleotide sequence of the β-tubulin gene from *A. flavus* and compare it with the β-tubulin gene from *A. nidulans*. This is the first gene sequence reported from the plant-pathogenic, aflatoxin-producing fungus *A. flavus*.

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MATERIALS AND METHODS

Fungal strains and culture. *A. flavus* 774 (*white pdx afl-21*) (ATCC 60045) was obtained from the collection of K. E. Papa (2, 20). The *pyr* mutation was introduced into strain 774 by parasexual transfer (20, 24), and the resulting strain was designated 774-2 (*white pdx afl-21 pyr*). *A. flavus* CRA01-2B (CRA) was obtained from UV-irradiated conidia of the aflatoxin-producing strain NRRL 3357 (Northern Regional Research Center, Peoria, Ill.) and is resistant to MBC [2-(methoxycarbonylamino)benzimidazole; a gift of Du Pont, Wilmington, Del.], the active component of the fungicide benomyl, at 50 μg/ml.

Potato dextrose agar, potato dextrose broth, and Czapeck's solution agar (Difco Laboratories, Detroit, Mich.) were used to culture and maintain *A. flavus* strains. Media were amended with uracil (10 mM) and pyridoxine (100 μg/ml) when necessary. MBC was added to media from a stock solution in dimethyl sulfoxide. Cultures were incubated at 36°C.

Bacterial DNA preparation techniques. *Escherichia coli* DH1 and JM109 were used in this study. Bacterial culture, transformations, and DNA preparations were done by standard methods (10).

Fungal DNA isolation. High-molecular-weight DNA (>150,000) was isolated from the benomyl-resistant strain, CRA, and from transformants by using a cetyltrimethylammonium bromide method described by Murray and Thompson (16). The DNA from these strains was purified by centrifugation in cesium chloride.

Construction and screening of the genomic library. Plasmid

pRG3 was a gift from G. S. May, Baylor University. Cosmid pCW10 was constructed from pRG3 as described previously (24). The genomic library construction essentially followed the procedures used in construction of cosmid libraries previously published for other filamentous fungi (23, 25). Insert DNA was prepared by partial digestion of the *A. flavus* DNA with *Sau*3A. The DNA (500 μg) was fractionated on a 1 to 5 M NaCl gradient and centrifuged in a Beckman SW-28 rotor at 25,000 rpm for 12 h. The region of the gradient containing DNA ranging in size from 35 to 45 kb was used for insertion into pCW10. The cosmid vector pCW10 was digested to completion with *Bam*HI followed by dephosphorylation with calf intestinal alkaline phosphatase. Insert DNA (2 μg) was ligated overnight with 3 μg of dephosphorylated vector DNA (vector/insert molar ratio, 9:1) in 20 μl. A 2-μl sample of the ligation mixture was treated with a lambda packaging extract. The packaged cosmids were used to transfect *E. coli* DH1 as described previously (10), resulting in 4.8×10^4 ampicillin-resistant colonies. A random screening of 10 of these colonies indicated an average insert size of approximately 40 kb. A total of 3,168 clones were preserved in 96-well microtiter plates at −80°C.

The ordered DNA library was screened on nitrocellulose filters by using a 96-well dot blot apparatus and standard methods of colony hybridization. A 5.1-kb *Pst*I fragment from pBA33 (a gift of G. S. May) containing the *benA* gene from *A. nidulans* was used as a probe to screen the library.

Fungal transformation. Freshly isolated protoplasts of *A. flavus* 774-2 were transformed by using a 50% polyethylene glycol 8000 solution (50% polyethylene glycol 10 mM Tris [pH 7.5], 0.6 M KCl, 50 mM $CaCl_2$) as described previously (24). Transformed protoplasts of *A. flavus* were resuspended in regeneration agar (Czapeck's solution agar containing 0.4 M ammonium sulfate, 100 μg of pyridoxine per ml, 1% agar). Single conidia were isolated from the transformants and grown on medium lacking uracil. To test for resistance to MBC, conidia were transferred to potato dextrose agar containing uracil and various concentrations of MBC. Transformed protoplasts were also directly selected for resistance to MBC by resuspending the protoplasts in 10 ml of regeneration agar containing 10 mM uracil and a range of MBC concentrations from 0.8 to 2.5 μg/ml.

Fungal hybridization analysis. Fungal DNA was digested to completion by restriction endonucleases, electrophoresed through 0.8% agarose, and transferred to nitrocellulose filters. Filters were hybridized with a 1.8-kb *Sal*I-*Eco*RI fragment of the *benA* gene from pBA33 or a 2.1-kb *Kpn*I fragment from pBRG4.

DNA sequencing. Clones for sequencing were constructed in M13mp18 and M13mp19 (17). Nested deletion subclones were produced by using the Cyclone 1 Biosystem (International Biotechnologies Inc., New Haven, Conn.).

DNA sequencing was performed by the dideoxy-chain termination method (21) by using the Kilobase system (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.). The sequences of both strands were determined. Sequence analyses were performed with the IBI DNA/Protein Sequence Analysis Computer software (International Biotechnologies Inc., New Haven, Conn.) and the DNA Inspector II software (Textco, West Lebanon, N.H.).

Nucleotide sequence accession number. The DNA sequence has been given the GenBank accession number M38265.

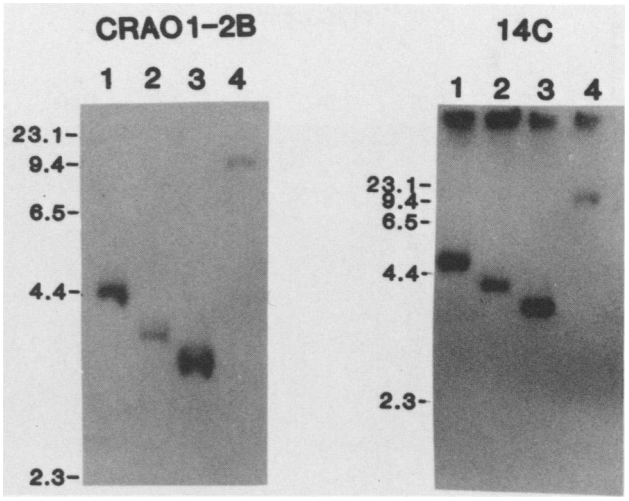

FIG. 1. Southern blot analysis of CRA genomic DNA and DNA from cosmid clone 14C. DNA was digested with restriction endonucleases *Pst*I (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), or *Eco*RI (lane 4). The molecular weight marker at the left of the blots is *Hin*dIII-*Eco*RI-digested lambda DNA. Blots were probed with a ^{32}P-labeled 1.8-kb fragment of the *benA* gene from *A. nidulans*.

RESULTS

Isolation and subcloning of the *A. flavus* β-tubulin gene. The genomic library of *A. flavus* was screened with the *benA* gene from *A. nidulans*. Of 1,920 cosmid clones tested, one (designated 14C) hybridized strongly to the *benA* probe. Restriction digests of genomic DNA from the benomyl-resistant mutant CRA and the cosmid 14C resulted in a single band of hybridization, indicating that the region of homology to the *benA* fragment lacks internal restriction sites for these enzymes (Fig. 1). The similar pattern of hybridization found with the genomic DNA and the cosmid DNA indicates that the correct flanking sequences are present in the cloned β-tubulin gene.

A 3.4-kb *Hin*dIII-*Pst*I fragment from cosmid 14C, containing the β-tubulin gene, was subcloned into plasmid pRG3, a transformation vector for *A. flavus* (24) which contains the selectable marker *pyr-4*. The resulting plasmid, pBRG4, was used in further transformation experiments and is shown in Fig. 2.

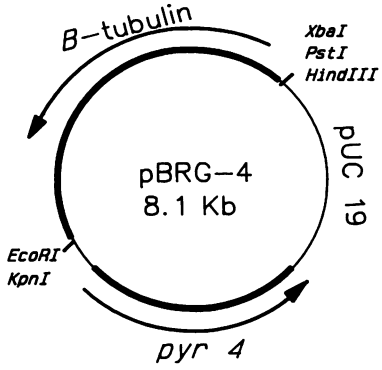

FIG. 2. Plasmid vector pBRG4 containing the altered β-tubulin gene from *A. flavus* CRA and the selectable marker *pyr-4*.

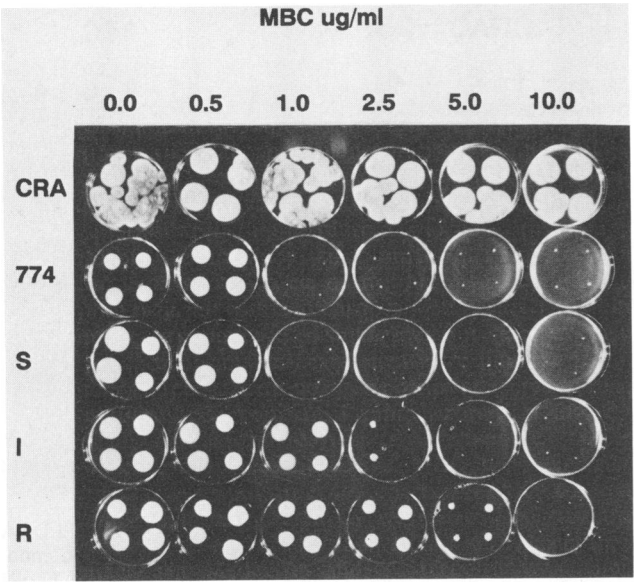

FIG. 3. Conidia after 4 days on potato dextrose agar amended with uracil and various concentrations of MBC, showing the resistant mutant CRA, the recipient strain 774-2, and transformants representing the sensitive (S), intermediate (I), and resistant (R) phenotypes. The right and left halves of each plate contain different individual transformants; two transformants are shown on each plate.

Transformation and expression of the *A. flavus* β-tubulin gene. *A. flavus* 774-2 was transformed by plasmid pBRG4, and transformants were selected on medium lacking uracil. The efficiency of transformation (3 transformants per μg of DNA) was similar to that of plasmid pRG3 (24). Of the 30 transformants examined for resistance to MBC, expression of resistance varied and could be grouped into three discrete phenotypes (Fig. 3). Six transformants were as sensitive as the recipient strain and would not grow on medium containing 1.0 μg of MBC per ml. Nineteen were intermediate in expression of resistance and grew on medium containing either 1.0 μg of MBC per ml (12 transformants) or 2.5 μg of MBC per ml (7 transformants). The five remaining transformants were resistant to 5.0 μg of MBC per ml. These transformants were cultured several times over a 2-year period, and each maintained its respective resistance to MBC.

Transformants were also selected directly on medium containing MBC. The concentration of MBC that allowed clear identification of MBC transformants varied among experiments, but was in the range of 1.2 to 1.6 μg of MBC per ml (Fig. 4). The average efficiency of transformation with pBRG4 was 1.3 colonies per μg of transforming DNA, lower than the 3 colonies per μg seen with uracil selection. Fifty-three colonies selected from two separate experiments were subcultured on 0.75 μg of MBC per ml and transferred to a range of concentrations of MBC. All colonies grew on 1.0 μg of MBC per ml, 43 grew on 1.2 μg/ml, 27 grew on 2.5 μg/ml, and 7 grew on 5.0 μg/ml. Compared with transformants selected for *pyr* complementation, colonies selected on MBC were more resistant to MBC. Whereas only 40% of colonies selected for *pyr* complementation grew at concentrations of 1.0 μg of MBC per ml or greater, 80% of the colonies selected on MBC grew at 1.0 μg of MBC per ml or greater. The lower apparent transformation efficiency on MBC may be due to failure to select less resistant colonies.

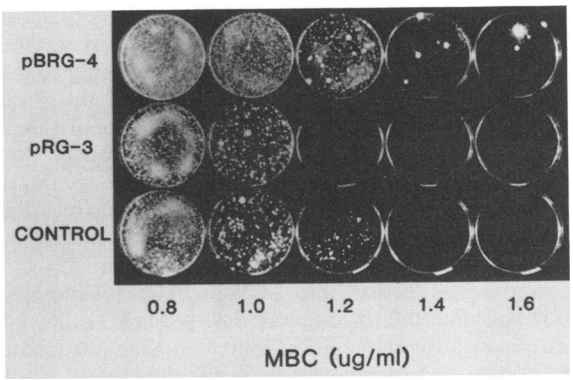

FIG. 4. Selection of MBC-resistant transformants on medium containing a range of MBC concentrations. Protoplasts were transformed with either pBRG4, a vector containing the altered β-tubulin gene of *A. flavus*, or pBRG3, a plasmid lacking the altered β-tubulin gene. Control reactions contained no DNA.

Hybridization analysis of transformants. Hybridization analysis of DNA from the benomyl-resistant mutant (CRA), recipient strain 774-2, and transformants representing each of the phenotypes S (sensitive to 1.0 μg of MBC per ml), I (resistant to 1.0 to 2.5 μg/ml), and R (resistant to 5.0 μg of MBC per ml) is shown in Fig. 5. The native β-tubulin gene appears as a 4.7-kb *Pst*I fragment on the blots. If there were integration of the transforming plasmid into the native site, one would expect disruption of the original fragment size. None of the transformants examined appeared to have integration at the site of the native β-tubulin gene. In the three MBC-resistant transformants, pBRG4 integrated at multiple sites in the genome. These transformants also had

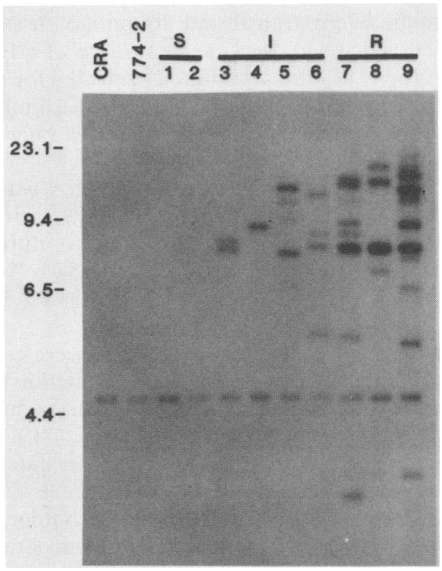

FIG. 5. Southern blot analysis of the benomyl-resistant mutant CRA, recipient strain 774-2, and transformants representing the sensitive (S), intermediate (I), and resistant (R) phenotypes. Genomic DNA was digested to completion with *Pst*I and probed with a 2.1-kb *Kpn*I fragment from pBRG4. Molecular weight markers at left depict the location of *Hin*dIII-digested lambda DNA.

tandem insertions of the plasmid. This was apparent because pBRG4 has only one *Pst*I restriction site and transformants with multiple tandem copies of pBRG4 show an 8.1-kb fragment on the Southern blot. The transformants that were intermediate in resistance had fewer copies of the plasmid than did resistant transformants, and no copies of the plasmid were evident in the two sensitive transformants.

In digests of CRA there was faint hybridization to a fragment at 8.2 kb with the 2.1-kb *Kpn*I fragment of pBRG4 or with a 0.65-kb *Kpn*I-*Bam*HI fragment from the coding region of the β-tubulin gene. This band was not present in strain 774 or strain 3357, the progenitor of CRA (data not shown).

Sequence of the β-tubulin gene. We sequenced 2.182 kb of the 3.4-kb insert in pBRG4 (Fig. 6). The amino acid sequence of β-tubulin from *A. flavus* has not been determined; therefore, the coding region of the β-tubulin gene was located by using the published nucleotide and amino acid sequences of the *benA* gene of *A. nidulans*. An open reading frame was identified with 98% amino acid homology to the *benA* gene of *A. nidulans*. A comparison of the remaining amino acids with those of *benA* showed R-group similarity (7) in all but three. Seven introns (IVS1 to IVS7) were predicted in the *A. flavus* gene ranging in size from 53 to 128 bp. The positions of these introns are identical to those in *A. nidulans*; however, intron 6 of *A. nidulans* is absent in *A. flavus*. Five introns (IVS1 through IVS5) are concentrated in the 5′ third of the coding region, and two (IVS6 and IVS7) are located in the 3′ third. Because this is the first sequenced gene from *A. flavus*, no consensus sequence has been established for the intron splice sites. However, analysis of the seven predicted 5′ and 3′ splice sites (Fig. 7) indicates that these sites conform to the GT-AC rule (14), and the splice sites are in agreement with the established consensus sequences of other fungi (9, 13, 19). It is apparent from the sequence data that the *A. flavus* β-tubulin gene contains one more amino acid than does the *benA* gene. An additional amino acid (leucine) is present at the beginning of the last exon.

The entire sequenced region from *A. flavus* has a G+C content of 54%; the G+C content is 57% in the coding region and 47% in the introns. Again, this is very similar to the *benA* gene of *A. nidulans*, which as a G+C content of 56.8% in the coding region and 46% in the introns (13). The *A. flavus* β-tubulin gene codes for a translation product of 448 amino acids with a molecular weight of 50,016. Codon usage in the *A. flavus* β-tubulin gene is biased; only 42 of 61 triplets are used (Table 1). This supports the hypothesis that genes expressed at high levels, such as the β-tubulin gene, have a more limited codon usage than genes expressed at low levels (3).

Sequence analysis of the 5′ noncoding region of the *A. flavus* β-tubulin gene does not reveal TATA box or CAAT-like regions, although there is a pyrimidine-rich region at bp −147 to −114 upstream from the initiation codon. Additionally, the AUG initiation codon is preceded by the sequence CCACA, which agrees with the consensus sequence (C/T)(A/C)ACA reported for *Aspergillus* species (1, 8). The sequence of the region 3′ to the termination codon does not show the AATAAA polyadenylation signal typical of higher eucaryotes; however, this signal is not well conserved in other filamentous fungi (1).

DISCUSSION

The objective of this study was to determine whether a homologous β-tubulin gene could be used as a selectable marker for the transformation of *A. flavus*. Because the β-tubulin gene is the first gene isolated and sequenced from *A. flavus*, it was also of interest to make a comparison of the gene with that in *A. nidulans*. The gene was isolated from a cosmid obtained from a DNA library of an MBC-resistant strain of *A. flavus*. A vector was constructed containing both the altered β-tubulin gene from *A. flavus* and the *pyr-4* gene from *N. crassa*. Transformants were selected by resistance to MBC or by uracil independence. The efficiency of transformation was dependent on the method of selection; direct selection on MBC was two to three times lower than when selection was for uracil independence. The apparent reason for the lower efficiency was that transformants expressing low or intermediate levels of resistance failed to grow on the concentration of MBC used in the regeneration medium. This observation is supported by the analysis of 30 clones selected for uracil independence. When subsequently grown on various concentrations of MBC, only 40% of the colonies selected for *pyr* complementation grew at concentrations of MBC greater than 1.0 μg/ml. In contrast, 80% of colonies selected on MBC grew at concentrations greater than 1.0 μg/ml MBC.

Variation in the expression of benomyl resistance among *A. flavus* transformants appears to be due to the copy number of the β-tubulin gene integrated into the genome (Fig. 5). The more resistant transformants had multiple copies of the β-tubulin gene incorporated into multiple sites in the genome. Transformants intermediate in resistance had fewer copies of the altered β-tubulin gene, and the two sensitive transformants had no apparent copies. The lack of copies in the sensitive strains was surprising since they grew on medium lacking uracil, indicating that they contained the *pyr-4* gene of plasmid pBRG4. Possibly the altered β-tubulin gene was excised from the genome. It is interesting that no transformants were identified in which the altered β-tubulin integrated at its homologous site. Either this did not occur or this type of integration was lethal. Transformation of *A. nidulans* with a plasmid containing both *pyr-4* and a native β-tubulin gene resulted in integration into the homologous β-tubulin DNA 67% of the time (12). Yelton et al. (25), however, found that transformation of *A. nidulans* with a native *trpC* gene results in heterologous integration.

None of the transformants examined were as resistant to MBC as was CRA (the source of the altered β-tubulin gene). The reason for this observation is not clear. Dunne and Oakley (5) reported that transformants of *A. nidulans* intermediate in resistance to MBC were the result of simultaneous expression of the native gene and the integrated resistance gene. This could be the case with *A. flavus*, as Southern analysis revealed that all transformants possessed a *Pst*I fragment that contained the wild-type gene. Other reasons for reduced resistance are possible. Unfortunately, the precise genetic nature of the resistance in strain CRA cannot be determined easily, because no sexual cycle is known in *A. flavus* and this strain does not have suitable markers for parasexual analysis. It is possible that the isolated β-tubulin gene is only partially responsible for the resistance observed in strain CRA.

Mullaney and Klich (15) found a high degree of hybridization between the *tubC* gene of *A. nidulans* and *Eco*RI fragments of *A. flavus*, *A. ochraceus*, *A. niger*, *A. terreus*, and *A. fumigatus*. We found a high degree of similarity between the β-tubulin gene of *A. flavus* and the *benA* gene of *A. nidulans*. There is 98% homology between the predicted amino acid sequences of the *A. flavus* β-tubulin gene and the *benA* gene of *A. nidulans*. Furthermore, the positions of the

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   1 CCCCTTGACGCCCGCACAACGAACAACTTGACGTTCCTCACCGCTCAACTTCAAGGCCATCTTTCCTCCTTCTCTCTTCT

  81 CCTCTTCCTTTTACCTACTCCCCGTCGACTGTCTCCCCCAGTCTATCCAACAACCCTTCTCCAACGACCTCTTCGCCGTT

 161 TTCAAACCCACCTTTTCCTACCAACAACGCCAAAATCCCCTCCACAATGCGTGAGATCGTATGTTGCTCCCTACCCCCGG
                                                  M  R  E  I                          4
 241 TGGGGGGAGAAGTCTGCTCAAAAAGCCCTATCCCCCCCCCCCTGATAGGGACCCCACCCGTTCTCCAATACTACAAGGTTG

 321 CTGACGGAGTTTGTTTCATCATATAGGTTCACCTTCAGACCGGCCAGTGTGTAAGTTCGACTATGATTTGATGTCTAGCA
                                  V  H  L  Q  T  G  Q  C                             12
 401 GGACCATGGCGACGGATACTAAACGTATGTTGGTGATAGGGTAACCAAATAGGTGCCGCTTTCTGGTATGTCTCAATGCC
                                                  G  N  Q  I  G  A  A  F  W          21
 481 TTCGAGTTAGTATGCTTTGGACCAAGGAACTCCTCAAAAGCATGATCTCGGATGTGTCCTGTTATATCTGCCACATGTTT

 561 GCTAACAACTTTGCAGGCAAACCATCTCTGGCGAGCACGGCCTTGACGGCTCCGGTGTGTAAGTACAGCCTGTATACACC
                        Q  T  I  S  G  E  H  G  L  D  G  S  G  V                    35
 641 TCGAACGAACGACGACCATATGGCATTAGAAGTTGGAATGGATCTGACGGCAAGGATAGTTACAATGGCTCCTCCGATCT
                                                                 Y  N  G  S  S  D  L  42
 721 CCAGCTGGAGCGTATGAACGTCTACTTCAACGAGGTGCGTACCTCAAAATTTCAGCATCTATGAAAACGCTTTGCAACTC
      Q  L  E  R  M  N  V  Y  F  N  E                                               53
 801 CTGACCGCTTCTCCAGGCCAGCGGAAACAAGTATGTCCCTCGTGCCGTCCTCGTTGATCTTGAGCCTGGTACCATGGACG
                     A  S  G  N  K  Y  V  P  R  A  V  L  V  D  L  E  P  G  T  M  D   74
 881 CCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGTGCTGGTAACAACTGG
    A  V  R  A  G  P  F  G  Q  L  F  R  P  D  N  F  V  F  G  Q  S  G  A  G  N  N  W 101
 961 GCCAAGGGTCACTACACCGAGGGTGCCGAACTTGTTGACCAGGTTGTCGATGTTGTCCGTCGCGAGGCTGAGGGCTGCGA
      A  K  G  H  Y  T  E  G  A  E  L  V  D  Q  V  V  D  V  V  R  R  E  A  E  G  C  D 128
1041 CTGCCTCCAGGGTTTCCAGATTACCCACTCCCTCGGTGGTGGTACCGGTGCCGGTATGGGTACTCTCCTGATCTCCAAGA
      C  L  Q  G  F  Q  I  T  H  S  L  G  G  G  T  G  A  G  M  G  T  L  L  I  S  K  154
1121 TCCGTGAGGAGTTCCCCGACCGTATGATGGCCACCTACTCCGTTGTCCCCTCCCCCAAGGTCTCCGACACCGTTGTTGAG
    I  R  E  E  F  P  D  R  M  M  A  T  Y  S  V  V  P  S  P  K  V  S  D  T  V  V  E 181
1201 CCCTACAACGCCACTCTTTCCGTCCACCAGCTTGTTGAGCACTCCGACGAGACCTTCTGTATCGACAACGAGGCTCTGTA
      P  Y  N  A  T  L  S  V  H  Q  L  V  E  H  S  D  E  T  F  C  I  D  N  E  A  L  Y 208
1281 TGACATTTGCATGCGCACCCTCAAGCTCTCCAACCCCTCTTACGGTGACCTGAACCACCTGGTCTCTGCTGTCATGTCTG
      D  I  C  M  R  T  L  K  L  S  N  P  S  Y  G  D  L  N  H  L  V  S  A  V  M  S  234
1361 GCGTGACCACCTGTCTCCGTTTCCCCGGTCAGCTCAACTCTGATCTTCGCAAGTTGGCCGTCAACATGGTTCCTTTCCCT
    G  V  T  T  C  L  R  F  P  G  Q  L  N  S  D  L  R  K  L  A  V  N  M  V  P  F  P 261
1441 CGTCTTCACTTCTTCATGGTTGGCTTCGCTCCTCTGACCAGCCGCGGTGCCCACTCTTTCCGTGCCGTCTCCGTTCCTGA
      R  L  H  F  F  M  V  G  F  A  P  L  T  S  R  G  A  H  S  F  R  A  V  S  V  P  E 288
1521 GTTGACCCAGCAGATGTTCGACCCCAAGAACATGATGGCTGCTTCTGACTTCCGTAACGGTCGTTACCTCACCTGCTCTG
      L  T  Q  Q  M  F  D  P  K  N  M  M  A  A  S  D  F  R  N  G  R  Y  L  T  C  S  314
1601 CTATCTTGTGATGTGGCCCCTATTTTCTATTTGTTCTATCCTCTGTTGTTTGAAAACTGACCTTTCGATAGCCGCGGAAA
    A  I  F                                                                 R  G  K 320
1681 GGTCTCCATGAAGGAGGTTGAGGACCAGATGCGCAACATCCAGAGCAAGAACCAGACCTACTTCGTCGAGTGGATCCCCA
      V  S  M  K  E  V  E  D  Q  M  R  N  I  Q  S  K  N  Q  T  Y  F  V  E  W  I  P  346
1761 ACAACATCCAGACCGCCCTGTGCTCCATTCCTCCCCGTGGTCTCAAGATGTCCTCCACCTTCATTGGAAACTCCACCTCC
    N  N  I  Q  T  A  L  C  S  I  P  P  R  G  L  K  M  S  S  T  F  I  G  N  S  T  S 373
1841 ATCCAGGAGCTCTTCAAGCGTGTCGGCGACCAGTTCACTGCTATGTTCCGTCGCAAGGCTTTCTTGCATTGGTACACTGG
      I  Q  E  L  F  K  R  V  G  D  Q  F  T  A  M  F  R  R  K  A  F  L  H  W  Y  T  G 400
1921 TGAGGGTATGGACGAGATGGAGTTCACTGAGGCTGAGAGCAACATGAACGACCTTGTCTCCGAGTACCAGCAGTACCAGG
      E  G  M  D  E  M  E  F  T  E  A  E  S  N  M  N  D  L  V  S  E  Y  Q  Q  Y  Q  426
2001 ATGCCTCCATCTCCGAGGGCGAGGAGGAATAGTAAGGATTCCCATTGGCCCTGCTCTCGTGTATTTGTGCTAACCAGTTT
    D  A  S  I  S  E  G  E  E  E  Y                                               437
2081 GCAGCCTCGAGGAGGAGGAGCCCCTTGAGCACGAGGAGTAAATAGCTTCCAGTCACTAAAGACTCGGATTGATATCTGGC
         *L  E  E  E  E  P  L  E  H  E  E  X                                       448
2161 AGCAATACCCTTGATAAGTCCA
```

FIG. 6. Nucleotide sequence of the noncoding strand and predicted amino acid sequence of the β-tubulin gene from *A. flavus*. Numbers in the left-hand margin indicate the position of the first nucleotide in each line; numbers in the right-hand margin indicate the position of the last amino acid in each line. Examination of the 3′ splice site in IVS7 suggests that there is an additional amino acid present in *A. flavus* not present in *A. nidulans* (*).

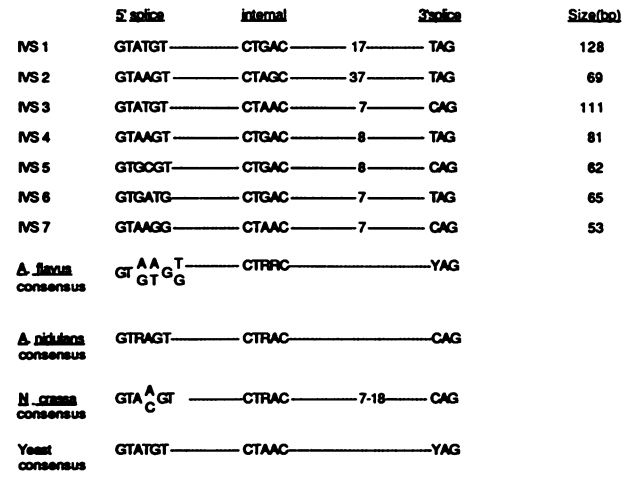

FIG. 7. Comparison of splice site sequences in the *A. flavus* β-tubulin introns with consensus sequences from *A. nidulans* (14), *N. crassa* (19), and *Saccharomyces cerevisiae* (9). Abbreviated forms of the introns are shown with the 5′, internal splice, and 3′ regions aligned. Distances in nucleotides between the internal and 3′ splice sequences are shown for all seven *A. flavus* introns. Purines are represented as R, and pyrimidines are represented as Y.

introns are highly conserved between the two β-tubulin genes. A notable difference is that *A. flavus* lacks intron 6 present in the *benA* gene; also, there is an additional leucine residue present at position 148 in the *A. flavus* β-tubulin gene

TABLE 1. Codon usage in the *A. flavus* β-tubulin gene

amino acid	codon	No. in β-tubulin	amino acid	codon	No. in β-tubulin
Phe	UUC	25 (100)[a]	Glu	GAA	2 (5)
	UUU	0 (0)		GAG	35 (95)
Leu	CUA	0 (0)	Ser	UCA	0 (0)
	UUA	0 (0)		AGC	4 (12)
	CUC	14 (41)		UCC	22 (65)
	CUG	7 (21)		UCG	0 (0)
	UUG	3 (9)		AGU	0 (0)
	CUU	10 (29)		UCU	8 (23)
Ile	AUA	1 (6)	Pro	CCA	0 (0)
	AUC	11 (69)		CCC	12 (63)
	AUU	4 (25)		CCG	0 (0)
Met	AUG	20 (100)		CCU	7 (37)
Val	GUA	0 (0)	Thr	ACA	0 (0)
	GUC	17 (52)		ACC	19 (79)
	GUG	2 (6)		ACG	0 (0)
	GUU	14 (42)		ACU	5 (21)
Tyr	UAC	12 (86)	Ala	GCA	0 (0)
	UAU	2 (14)		GCC	15 (56)
His	CAC	10 (91)		GCG	0 (0)
	CAU	1 (9)		GCU	12 (44)
Gln	CAA	2 (9)	Cys	UGC	5 (62.5)
	CAG	21 (91)		UGU	3 (37.5)
Asn	AAC	22 (96)	Trp	UGG	4 (100)
	AAU	1 (4)	Arg	AGA	0 (0)
Lys	AAA	0 (0)		CGA	0 (0)
	AAG	13 (100)		CGC	7 (30)
Asp	GAC	17 (77)		AGG	0 (0)
	GAU	5 (23)		CGG	0 (0)
				GGG	0 (0)
				CGU	16 (70)
			Gly	GGU	24 (63)
				GGC	11 (29)
				GGA	3 (8)

[a] The numbers in parentheses represent the percentage of the amino acid that is encoded by the codon.

that is absent in the *benA* gene of *A. nidulans*. A high degree of similarity between the β-tubulin genes from these two fungi may be expected, as the anamorphs of these fungi are in the same genus; however, the true relatedness of these fungi cannot be confirmed because no teleomorph is known for *A. flavus*. Typical of other genes in filamentous fungi, the introns of the *A. flavus* β-tubulin gene are relatively short and the majority are found in the 5′ third of the coding sequence. The splice site sequences for the 5′, internal, and 3′ regions of *A. flavus* contain pyrimidine-rich regions at bp −147 to −114. Other filamentous fungal genes have similar pyrimidine-rich regions which precede their transcriptional start sites as determined by S1 nuclease and primer extension analysis (1, 13, 19). The start site in the *A. flavus* gene may also be located near this region; however, this must be confirmed by further experimentation.

In *N. crassa*, a single thymine-to-adenine transversion resulting in a phenylalanine-to-tyrosine change at amino acid 167 has been reported to be responsible for resistance to benomyl (19). It is interesting that in the *A. flavus* β-tubulin gene conferring resistance to benomyl, the base at position 167 is also an adenine and the respective triplet codes for a tyrosine. This suggests that the mutation conferring resistance to benomyl in *N. crassa* may also confer resistance to benomyl in *A. flavus*.

The ability to use the altered β-tubulin gene allows us to transform isolates of *A. flavus* without prior mutation to auxotrophy. The low frequency of transformation limits the use of this selective marker for screening genomic libraries; however, the frequency is sufficient to determine whether specific genomic clones complement mutants blocked in the aflatoxin biosynthetic pathway. For example, we have identified genomic clones that hybridize to cDNAs prepared from mRNAs induced during aflatoxin biosynthesis (unpublished data). By using the β-tubulin transformation system, we can determine whether these clones complement the various pathway mutants.

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