

# Identification and Functional Analysis of Beta-Tubulin Genes by Site Specific Integrative Transformation in *Aspergillus nidulans*

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**ABSTRACT** We have cloned two different beta-tubulin sequences from the filamentous fungus *Aspergillus nidulans*. Each was used in the construction of transforming plasmids that carry the *pyr4* gene of *Neurospora crassa*. We used these plasmids to transform a *pyrG*<sup>-</sup> strain of *Aspergillus* to uridine prototrophy. Both plasmids were shown to integrate site specifically into the homologous chromosomal sequences. We then used transformant strains in genetic crosses to demonstrate that one of the cloned beta-tubulin sequences was the *benA* beta-tubulin gene, which codes for the beta1- and beta2-tubulins. The other cloned beta-tubulin sequence was shown to be the structural gene for beta3-tubulin by gene disruption and to participate in conidial development. This is the first report of a gene disruption by site specific, integrative recombination in *Aspergillus nidulans*.

Biochemical analysis of the tubulins of *Aspergillus nidulans* has suggested that there are two alpha-tubulin and two beta-tubulin genes (26). Two alpha-tubulin polypeptides, alpha1 and alpha3 are produced by the *tubA* gene (9). The third alpha-tubulin, alpha2, is the product of the *tubB* gene. Similarly, two of the beta-tubulin polypeptides, beta1 and beta2, are produced by the *benA* gene (20), and the third, beta3, is produced by the *tubC* gene. Genetic analysis has thus far identified mutations in only one alpha-tubulin (*tubA*) and one beta-tubulin gene (*benA*). The *benA* beta-tubulin gene was identified as a mutation resulting in resistance to the antimicrotubule drug benomyl. Several *benA* mutations were shown to contain two electrophoretically altered beta-tubulin polypeptides, designated beta1 and beta2. These same studies provided evidence for the existence of a third beta-tubulin polypeptide, beta3, which was unaltered in *benA* mutants (20, 26). The *tubCA* alpha-tubulin gene was identified as an extragenic suppressor mutation of a temperature-sensitive *benA* mutant (9). The *tubA1* mutation was shown to have two electrophoretically altered alpha-tubulin polypeptides, designated alpha1 and alpha3. A third alpha-tubulin, alpha2, was not a product of the *tubA* gene. The data clearly suggest the existence of two alpha-tubulin and two beta-tubulin genes in *Aspergillus*.

Recently several laboratories have reported that *Aspergillus*

auxotrophs can be transformed to nutritional prototrophy by exogenously added plasmid DNA bearing an appropriate nutritional marker (1, 23, 28). In addition, two of these reports demonstrated that transformation of the plasmids was integrative and in some cases site specific (23, 28). The development of a DNA-mediated transforming system for *Aspergillus* has made it possible for us to demonstrate directly the existence of multiple beta-tubulin genes. We report here the construction of integrative transforming plasmids which carry one of two beta-tubulin sequences from *Aspergillus* and the *pyr4* gene as a nutritional marker from *Neurospora crassa*, which complements *pyrG* mutants of *Aspergillus* (1). We demonstrate that these plasmids integrate site specifically into the homologous chromosomal DNA and we use them further in determining which of the two cloned beta-tubulin sequences is the *benA* beta-tubulin gene. In addition, we use an integrative transforming plasmid designed to disrupt the unidentified beta-tubulin gene to determine that the beta3 polypeptide is the product of this new beta-tubulin gene and that it functions in conidial development in *Aspergillus*.

## MATERIALS AND METHODS

*Aspergillus Culture Conditions and Genetics:* The *Aspergillus* strains used were G191 (*pyrG89*, *fwA1*, *mauA2*, *pabaA1*), GB13 (*pyrG89*,

*fwA1, benA15*), GB20 (*pyrG89, fwA1, benA22*), GB32 (*pyrG89, yA2, pabaA1, benA33*), BR02 (*yA2, benA33*), and FGSC4 (Glasgow wild-type). Diploid strains were LO82, a diploid between a *yA2, pabaA1* strain (R21), and a *wA3, pyrOA4* (R153); and L0117, a diploid between a *benA33, yA2*, (BR02), and R153. 0.5% yeast extract, 2% glucose, 2% agar, trace elements, was used as the solid medium and was supplemented with 10 mM uridine for growth of *pyrG89* strains. 0.5% yeast extract, 2% glucose, trace elements, was used for growth in liquid culture and was supplemented with 10 mM uridine for *pyrG89* strains. Transformation of strains with plasmid DNA was essentially as described (1), but we noted increased numbers of transformants when spores were germinated for 5–6 h before protoplasting and when 0.6 M KCl was included in the polyethylene glycol 8000 solution. The genetic methods and recovery of pyrimidine auxotrophs were as described (4, 15). Temperature-sensitive strains (GB32) were grown at 32 °C as the permissive temperature and 42 °C as the nonpermissive temperature. Conidial revertant phenotype was scored for on 0.5% yeast extract, 2% glucose, 2% agar, trace elements, containing 4.8 µg/ml of benomyl.

**Bacteria and Plasmids:** *Escherichia coli* K-12 strain JM83 was used to propagate plasmids (25). The plasmids pUC9 and pUC19 were used in the construction of transforming plasmids (11, 25).

**Plasmid Construction, DNA Purification, and Analysis:** Fragments to be ligated into plasmids were purified on either agarose or acrylamide gels, and then electroeluted onto dialysis membrane. Fragments to be ligated together were combined, extracted twice with phenol/chloroform/isoamyl alcohol (50:49:1), precipitated with ethanol, washed once in ethanol, dried, and dissolved in water at 65 °C at a final DNA concentration of 5–20 µg/ml. Ligations were done in the buffer specified by the supplier for 2–16 h at 12 °C. The DNA was transformed into competent cells that had been prepared by the calcium chloride procedure (8). The desired clones were identified by restriction digesting DNA from minipreparations (7). Large-scale plasmid preparations were by the method of Godson and Vapnek (6).

*Aspergillus* genomic DNA was prepared from squeeze-dried mycelium which was frozen and pulverized in liquid nitrogen. 1 g powdered mycelium was resuspended in 5 ml of 50 mM Tris-HCl, 0.1 M EDTA, pH 8.0, 2% Sarkosyl, heated to 65 °C for 30 min, and treated with 100 µg/ml proteinase K for 16 h, the heavy debris was sedimented at 800 g for 10 min, and the DNA was centrifuged to equilibrium on CsCl, ethidium bromide gradients for 48 h. The DNA was removed from the gradient; extracted once with water-saturated *n*-butanol to remove ethidium bromide, twice with phenol/chloroform/isoamyl alcohol (50:48:2), and three times with diethyl ether; and the residual ether was evaporated at 65 °C. Cesium chloride was removed by dialysis against a 2,000-fold excess of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. Genomic DNA was digested with restriction endonucleases using the manufacturer's recommended buffer conditions with a 5–10-fold excess of enzyme for at least 6 h. Digested DNA was run on agarose gels in 40 mM Tris-acetate, 2 mM EDTA, pH 8.0 and transferred to nitrocellulose as described by Southern (21). Nick translations were done using the method of Rigby et al. (17). Hybridizations were done at 65 °C as described (14). After hybridization filters were washed twice at 65 °C for 30 min in two-times-concentrated SSC, 0.5% Sarkosyl NL-30, and twice for 30 min at 65 °C in 0.1-times-concentrated SSC, 0.025% Sarkosyl NL-30.

**Gel Electrophoresis:** Two-dimensional gel electrophoresis was performed as described previously (26).

**Materials:** Restriction endonucleases, T4 DNA ligase, and agarose were obtained from either BRL or New England Biolabs. Alpha-[<sup>32</sup>Pd]CTP (400 Ci/mmol) was obtained from Amersham Corp. (Arlington Height, IL). DNA polymerase I was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), nitrocellulose BA85 from Schleicher & Schuell, Inc. (Keene, NH), and media components from either Difco Laboratories Inc. (Detroit, MI) or Sigma Chemical Co. (St. Louis, MO).

## RESULTS

### *Cloning of Beta-Tubulin Genes and Plasmid Construction*

Restriction maps for two beta-tubulin clones, previously selected from a Charon4A library (13), are shown in Fig. 1, *A* and *C*. Genomic blots probed with either the beta5 or beta14 clones showed no cross-hybridization under the conditions used (Fig. 2). The beta5 and beta14 sequences will cross-hybridize under conditions of lower hybridization stringency. This demonstrates that the beta5 and beta14 sequences hybridize with different DNA fragments and that two different

beta-tubulin sequences were cloned. A 5.4 kilobase (kb)<sup>1</sup> Pst I fragment of beta5 (Fig. 1 *C*) containing all of the beta-tubulin gene was subcloned into the Pst I site of pUC19 (11), and a 3.2-kb Xho I fragment of beta14 (Fig. 1 *A*) containing all but 12 amino terminal amino acids was subcloned into the Sal I site of pUC9. These plasmids were designated pB-5 and pB-14, respectively.

Construction of transforming plasmids was simplified by first subcloning the *pyr4* gene of *Neurospora crassa* from plasmid pFB6 as a 2.2-kb Bgl II, Pst I fragment into BamH I, Pst I-digested pUC9, to produce the plasmid pODC. We constructed a beta14 transforming plasmid by first ligating the 2.2-kb EcoR I, Pst I *pyr4*-containing fragment from pODC and the 3.2-kb EcoR I, Hind III fragment from pB-14 together into Pst I, Hind III-digested pUC9. The resulting plasmid harbored the *pyr4* gene flanked by EcoR I sites. This plasmid was designated AlpGM1 for *Aspergillus* integrative plasmid (Fig. 3). The plasmid nomenclature is adapted from that used for yeast transforming plasmids (22). We constructed the plasmid AlpGM4 by cloning the 2.2-kb EcoR I flanked *pyr4* gene from AlpGM1 into the EcoR I site of pB-5 (Fig. 3). The plasmid AlpGM6, which we used in integrative disruption experiments, was constructed by first subcloning the 750-base-pair BamH I, Pst I fragment from pB-14 into pUC19 and the *pyr4* from AlpGM1 into the EcoR I site of this plasmid (Fig. 3).

### *Site Specific Integration of AlpGM1 and AlpGM4 in Aspergillus*

We used plasmids AlpGM1 and AlpGM4 to transform the *pyrG*<sup>-</sup> G191 strain of *Aspergillus* to uridine prototrophy. The frequency of transformation was 2–4 transformants/µg plasmid DNA, similar to that reported for the parent plasmid pFB6 (1).

Site specific integration of the plasmids was demonstrated by Southern analysis of restriction endonuclease-digested total DNA from transformants. Integration of AlpGM1 by homologous recombination would be expected to generate a new restriction pattern for Xho I-digested transformant DNA, when probed with the insert of pB-14. If AlpGM1 integrated into the homologous sequence in the chromosome, the 3.2-kb Xho I band would be lost and two new bands generated, one of 4.7 kb and another of 6.6 kb (Fig. 1 *B*). We observed this predicted hybridization pattern in eight of nine transformants, which indicates that the plasmid had integrated into the homologous site in these eight transformants (Fig. 4 *A*). In three of the transformants an additional plasmid-sized band of 8.1 kb was observed as the result of multiple tandem integrations at the same site (Fig. 4 *A*, lanes 4, 5, and 7). We tested site specific integration of AlpGM4 using Southern blot analysis of EcoR I-digested transformant DNA probed with the insert from pB-5. The genomic beta5 gene is contained in an ~19-kb EcoR I fragment, and, since the subcloned fragment contains no EcoR I sites, digestion of transformant DNA with EcoR I should result in two bands of hybridization, whose total size should be the sum of the endogenous EcoR I fragment (19 kb) plus the plasmid sequences less the *pyr4* sequences (8.1 kb) for a total of 27.1 kb, if it integrated into the homologous chromosomal DNA (Fig. 1 *D*). The predicted bands would therefore be ~16 and 11 kb.

<sup>1</sup> Abbreviation used in this paper: kb, kilobase.

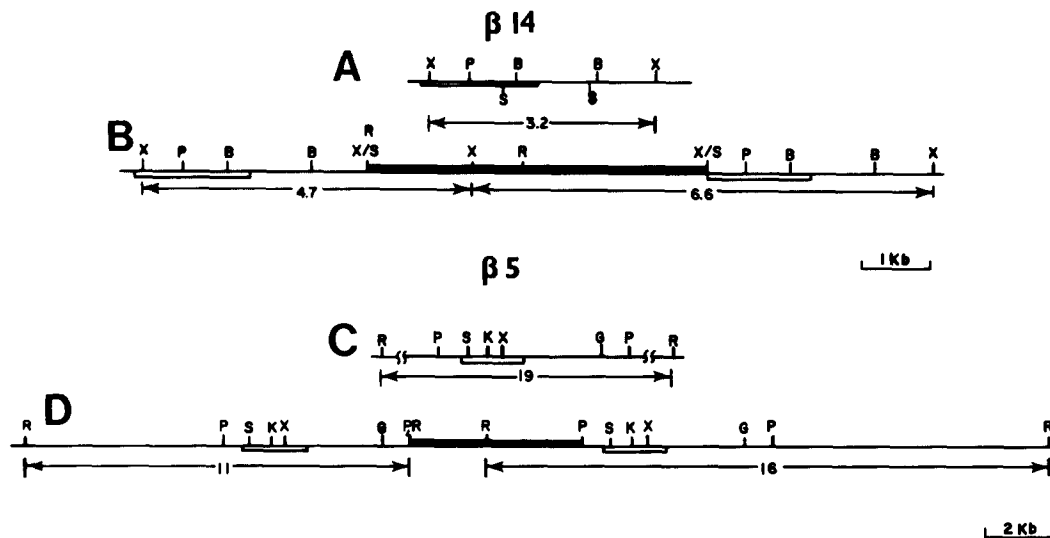


FIGURE 1 Restriction endonuclease maps of the beta14 clone (A) and the beta5 clone (C). Restriction maps were constructed using a combination of single and double enzyme digestions. The restriction enzymes used were BamH I (B), Bgl II (G), Kpn I (K), Pst I (P), Sac I (S), and Xho I (X). The open bar on each map represents the approximate limits of the coding region for each clone. The maps show the predicted chromosomal DNA structure for the beta14 (B) and beta5 (D) loci after integration of the plasmid AlpGM1; for the beta14 sequence; and of the plasmid AlpGM4, for the beta5 sequence. The open bar on the maps represents the approximate limits of the coding region for each gene and the filled bar represents the vector sequences contributed by the plasmids. Sites for cleavage by the restriction endonucleases BamH I, Bgl II, Eco RI, Kpn I, Pst I, Sac I, and Xho I are indicated in addition to the position of the Xho I, Sal I (X/S) fusion site generated during the construction of the plasmid AlpGM1. The size of predicted restriction fragments that should be detected by hybridization for transformants is indicated below each map. The beta-tubulin sequences used in this study were cloned from a Charon4A library (12) by hybridization to a chicken beta-tubulin cDNA (24) and cycles of plaque purification. Clones were positively identified as coding for beta-tubulins by partial sequence analysis (Tsang, M. L.-S., A. Upshall, G. S. May, S. A. Fidel, and N. R. Morris, manuscript in preparation) and comparison with published beta-tubulin sequences (10, 24).

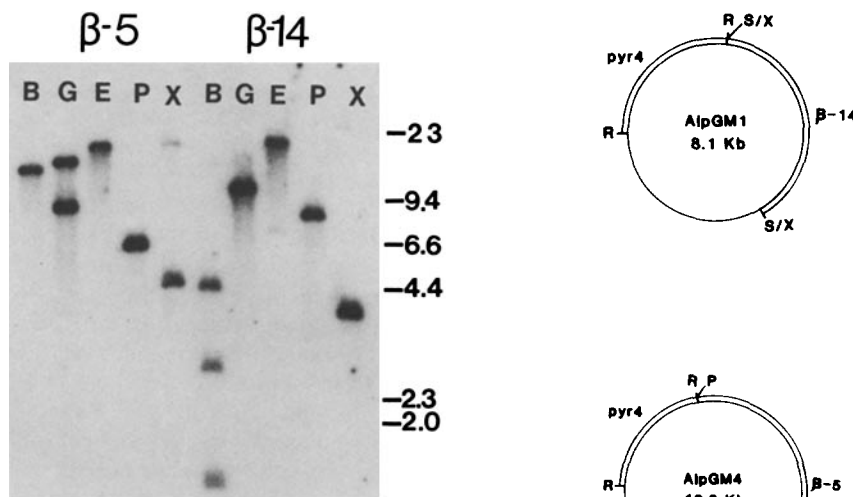


FIGURE 2 Hybridization of pB-5 and pB-14 inserts to total *Aspergillus* DNA. DNA (2  $\mu$ g) was digested with restriction endonucleases, fractionated on a 1% agarose gel, and transferred to nitrocellulose. The filters were hybridized with a nick-translated insert of pB-5 or pB-14. The restriction enzymes used were BamH I (B), Bgl II (G), Eco RI (E), Pst I (P), and Xho I (X). The position and size in kb of lambda-Hind III markers are indicated at the right.

The bands observed were 15.4 and 11.3 kb, in agreement with the predicted sizes. This was observed for six of the nine transformants analyzed (Fig. 4B, lanes 5-9). Of the remaining three, in one case the plasmid had integrated into the homologous chromosomal sequence and a second unidentified site (Fig. 4B, lane 2), and in two others the plasmid had integrated into another unknown site (Fig. 4B, lanes 1 and 3).

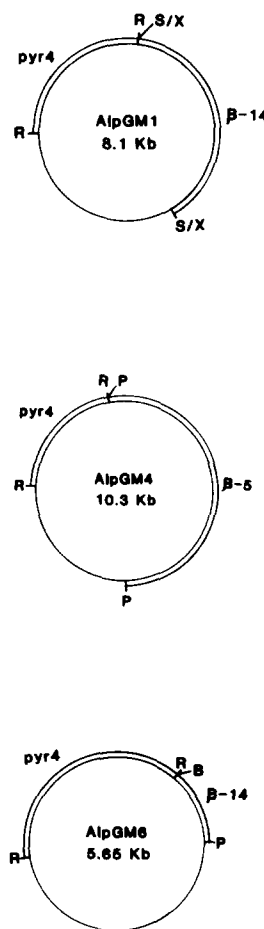


FIGURE 3 Structure of integrative transforming plasmids AlpGM1, AlpGM4, and AlpGM6. The size of the plasmids is indicated on each plasmid map. The positions of EcoRI (R) sites flanking the *pyr4* gene; the Sal I, Xho I (S/X) fusion sites for beta14 clone or the Pst I (P) sites for beta5 clone; and the BamH I (B) and Pst I (P) sites for the beta14 disrupter are indicated.

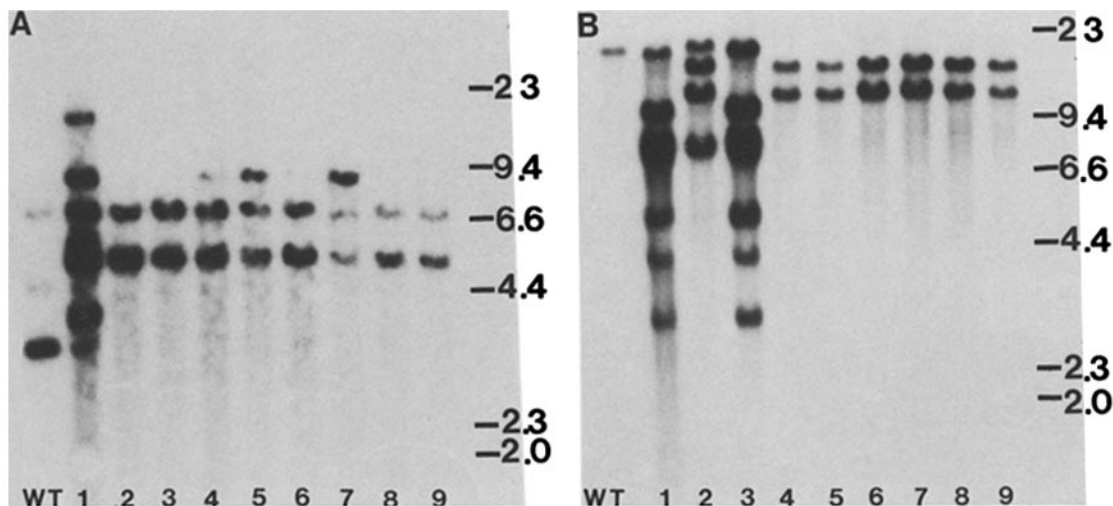


FIGURE 4 Southern analysis of total DNA from strains transformed with (A) AlpGM1 or (B) AlpGM4. *Aspergillus* transformant DNA (2  $\mu$ g) was digested with either Xho I for AlpGM1 transformants or Eco I for AlpGM4 transformants, fractionated on agarose gels, and transferred to nitrocellulose. The filter containing AlpGM1 transformant DNA was hybridized with a nick-translated insert from pB-14 and that with AlpGM4 transformant DNA was hybridized with a nick-translated insert from pB-5. Lane WT was total DNA from the nontransform wild-type parental strain and lanes 1–9 contain total DNA from transformants. The position and size in kb or lambda-Hind III markers are indicated at the right of each transfer.

### Identification of *benA* Beta-Tubulin

Strains that have the *pyr4* gene site specifically integrated can be used to identify which of the cloned beta-tubulin genes is *benA*. Using standard genetic methods, we tested the integrated *pyr4* gene for linkage to *benA* by analyzing the products of a cross between a *pyr4*<sup>+</sup> transformant and a *pyrG*<sup>-</sup>, *benA*<sup>r</sup> (benomyl resistant, GB13) strain. If *pyr4* was integrated at or near *benA* then little recombination should have been observed between the two genes, and *pyr4*<sup>+</sup> (uridine prototrophs) and *benA*<sup>r</sup> (benomyl resistant) recombinants should be rare and only the parental genotypes should be recovered from such a cross. This prediction was confirmed for the beta5 AlpGM4 transformants. The recombination frequency for *pyr4* at the beta5 gene and *benA* was ~1%, indicating that the beta5 gene was *benA*, whereas that for the beta14 gene was ~41%, indicating weak linkage between the integrated *pyr4* and *benA*. The 1% recombination frequency between the integrated *pyr4* and *benA* was not unexpected, since they are physically separated by the plasmid DNA. This clearly suggests that the beta5 beta-tubulin clone is the *benA* gene (Table I). This analysis also suggests that the beta14 gene may be located on chromosome 8 by its weak linkage to *benA*. The meiotic stability of the integrated *pyr4* marker was determined in the progeny from a “selfed” parental cleistothecium. The rate of marker loss was 7%, not enough to influence the linkage analysis.

Additional proof that the beta5 clone is *benA* was provided by an experiment in which the plasmid AlpGM4 was used to transform a strain (GB32) carrying a very heat-sensitive, benomyl-resistant mutation, *benA33*. The temperature-sensitive phenotype of *benA33* is suppressed in heterozygous diploids (*benA*/+), and these diploids are also sensitive to benomyl. Therefore, transformation of a *pyrG*<sup>-</sup>, *benA33* strain (GB32) with AlpGM4 might be expected to produce transformants with a phenotype like that of a *benA33*/+ heterozygous diploid. In contrast, transformation of the same strain with AlpGM1, which carries the beta14 gene, would not be expected to suppress the *benA33* mutation, since

TABLE I. Analysis of Linkage between *benA* and Either the beta14 Clone or the beta5 Clone Using the Integrated *pyr4* Gene

	beta14		beta5	
	<i>ben</i> <sup>r</sup>	<i>ben</i> <sup>s</sup>	<i>ben</i> <sup>r</sup>	<i>ben</i> <sup>s</sup>
<i>pyrG</i> <sup>-</sup>	114	74	102	6
<i>pyrG</i> <sup>+</sup>	77	105	2	90

Strains carrying the *pyr4* marker integrated at either the beta14 gene or the beta5 gene were crossed with GB13, a *pyrG*<sup>-</sup>, *benA*<sup>r</sup> (benomyl-resistant) strain, and the phenotype of the segregants was determined. The numbers represent the number of progeny with a given phenotype. Five beta14 transformants, representing 370 progeny from 11 cleistothecia, and three beta5 transformants, representing 200 progeny from 7 cleistothecia, were analyzed for linkage to *benA*.

mutations in *benA* suffice to give resistance to benomyl. 20 AlpGM1 and 20 AlpGM4 transformants were analyzed for temperature-sensitive growth. None of the AlpGM1 transformants showed any growth at 42°C, though they grew normally at 32°C, whereas 16 of the AlpGM4 transformants exhibited near-wild-type growth at both 42 and 32°C (Fig. 5). In addition, the strains transformed with AlpGM4 exhibited an intermediate resistance to benomyl (Fig. 6). The beta5 clone therefore can suppress the *benA33* mutation, further indicating that it is *benA*. The intermediate sensitivity of the beta5, AlpGM4, transformants to benomyl is interesting when you consider that diploids show near-wild-type sensitivity to benomyl (Fig. 6). This may be explained by a reduced expression of the transformed gene that only partially suppresses benomyl sensitivity while remaining sufficient to suppress the temperature sensitivity of the *benA33* gene. In addition, transformants with AlpGM4 in which the temperature sensitivity was not suppressed were still resistant to benomyl, which suggests an integrative event that has inactivated the cloned beta5 sequence (Fig. 6).

### What Are the Product and Function of the Beta14 Clone?

Since two beta-tubulin genes have been cloned and one, the beta5 clone, has been demonstrated to be *benA*, which is

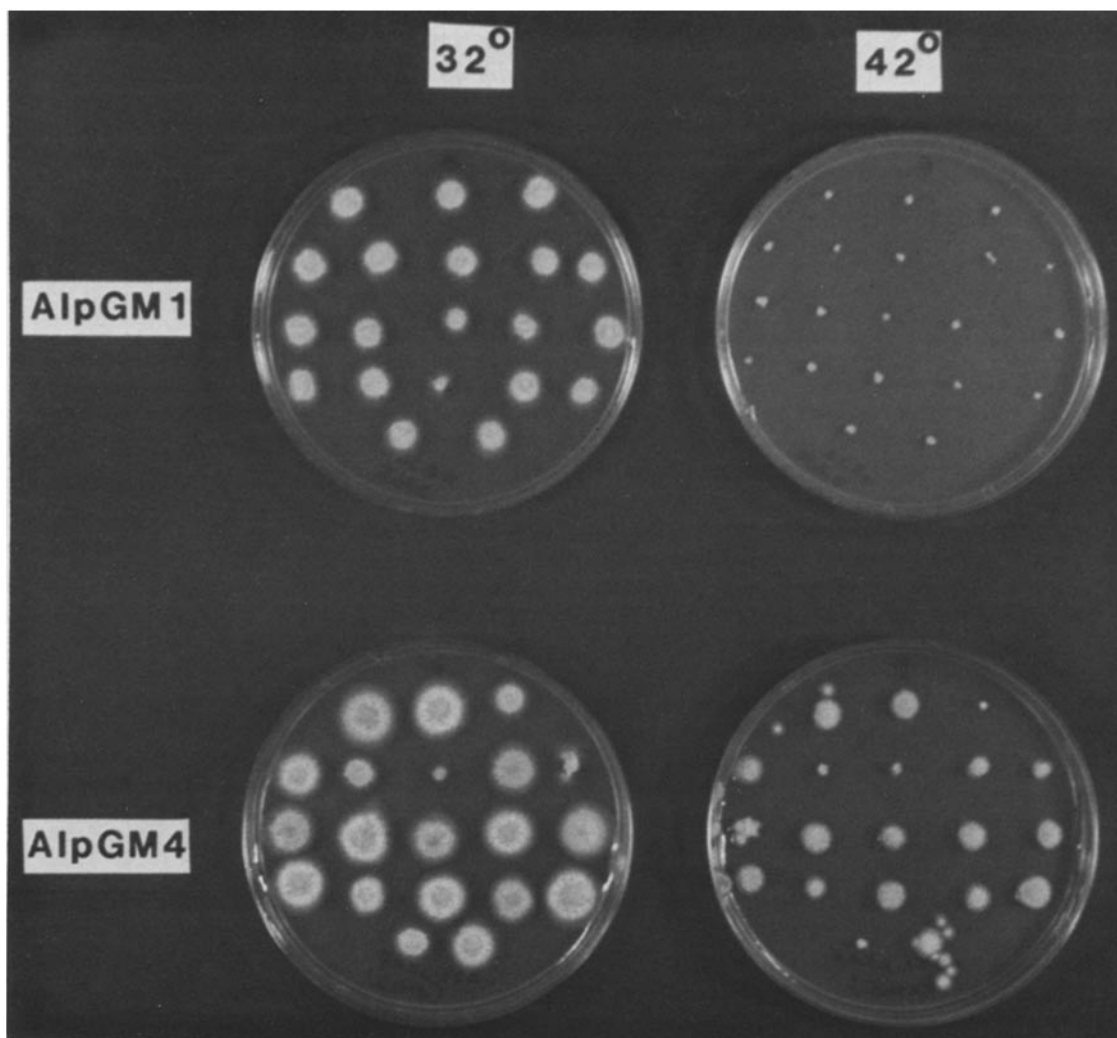


FIGURE 5 Suppression of the temperature-sensitive *benA33* allele by transformation with plasmid AlpGM4. Protoplasts of GB32 (*pyrG*<sup>-</sup>, *benA33*) were transformed to uridine prototrophy with either AlpGM1 or AlpGM4. Transformant colonies were transferred to duplicate plates. One plate was incubated at 32°C and the other was incubated at 42°C for 2 d. The plasmid AlpGM4 carrying the beta5 clone suppressed the temperature sensitive phenotype of *benA33* whereas AlpGM1, which carries the beta14 clone, did not.

the structural gene for beta1- and beta2-tubulins, it is reasonable to assume that the beta14 clone contains the structural gene for the beta3 polypeptide. Mutations in *benA* give rise to benomyl-resistant strains for vegetative growth, but these strains cannot form vegetative spores (conidia) in the presence of benomyl. This suggested that conidiation was a benomyl-sensitive process possibly involving another beta-tubulin gene. We tested this hypothesis by isolating mutations in a *benA*<sup>r</sup> (benomyl resistant) background that permitted conidiation in the presence of benomyl. These strains were designated CR<sup>-</sup> for conidial revertants and were examined for electrophoretically altered beta-tubulins.

20 conidial revertants were isolated and in all the beta3-tubulin was found to be absent and cosegregated with the CR<sup>-</sup> phenotype, suggesting that it is the beta3-tubulin which makes conidiation sensitive to benomyl (27). To determine that the beta14 clone was the structural gene for the beta3 polypeptide the resident chromosomal beta14 gene was disrupted by an integrative transforming plasmid, AlpGM6, in a *pyrG*<sup>-</sup>, *benA*<sup>r</sup> strain (GB20). Integration of AlpGM6 via homologous recombination into the chromosomal beta14

sequence would result in two incomplete and therefore non-functional copies of the beta14 gene. We reasoned that if the beta14 clone was the structural gene for the beta3 polypeptide then disruption of this gene in a benomyl resistant strain would result in the CR<sup>-</sup> phenotype. Initially we examined the DNA of five uridine prototrophic transformants for disruption of the beta14 gene. In only one of the five did we observe disruption of the resident beta14 gene, and it was the only one which was observed to give the CR<sup>-</sup> phenotype on benomyl. We subsequently analyzed 150 uridine prototrophic transformants for the CR<sup>-</sup> phenotype and obtained a total of nine CR<sup>-</sup> isolates, 6% of all the transformants analyzed. The frequency of site specific integration was not unexpected considering the small size of the beta14 bearing DNA fragment carried on AlpGM6. The total number of transformants we obtained using this plasmid was similar to that we obtained using the others. All of these were both CR<sup>-</sup> and had the resident beta14 gene disrupted. These strains grew normally and produced conidia in the presence of benomyl, as expected of true conidial revertants. Southern analysis of total DNA from seven of these transformants showed that in each the

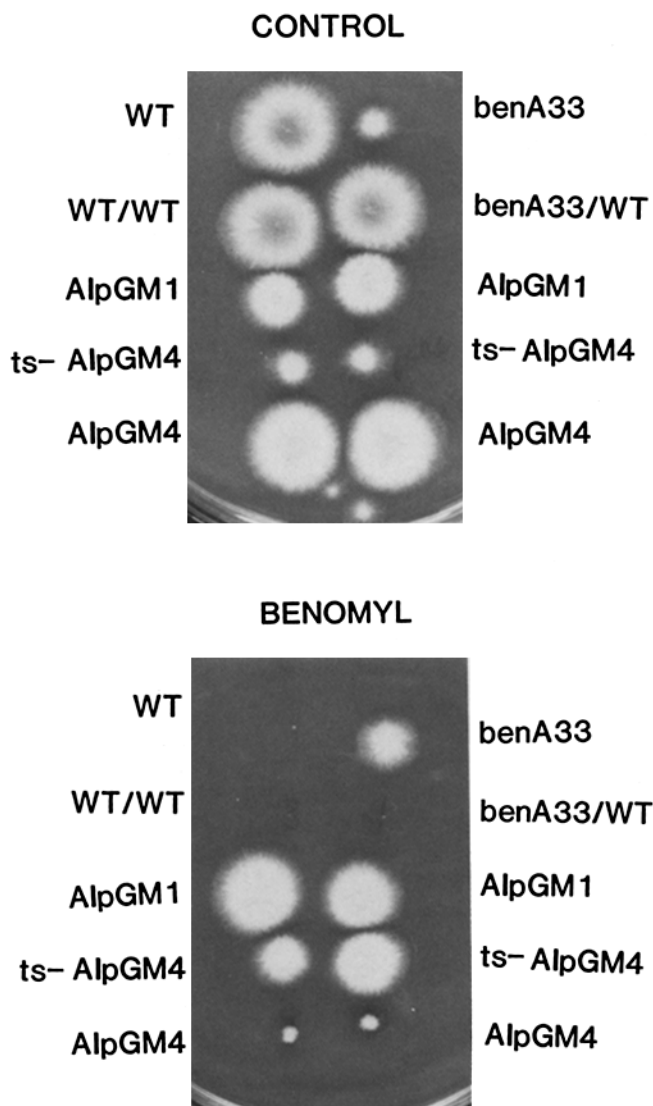


FIGURE 6 Benomyl sensitivity of strains carrying different beta-tubulin complements. Haploid strains were FGSC4 (*WT*), BRO2 (*benA33*,  $\gamma$ A2, *pabaA1*). Diploid strains were LO82 (*WT/WT*), LO117 (*benA33/WT*), and transformant strains carrying AlpGM1, which are *ts*<sup>-</sup> and resistant to benomyl; AlpGM4, which were *ts*<sup>-</sup> (*ts*<sup>-</sup>AlpGM4) and benomyl resistant, as demonstrated in this experiment; and AlpGM4, which are not temperature sensitive and as shown here are sensitive to benomyl. The reduced growth of *benA33* strain in the absence of benomyl at 32°C is because even at this temperature growth is inhibited. In addition, the temperature sensitivity is suppressed in the presence of benomyl (11).

homologous chromosomal sequence had been disrupted (Fig. 7A). The band of variable intensity at 3.45 kb in each of the transformants would be expected if multiple tandem integrations had occurred, with the intensity of the band being proportional to the number of copies integrated. Analysis of the beta-tubulin polypeptides from each of the transformants shows that they also lack the beta3 polypeptide (Fig. 7B). To be certain that the CR<sup>-</sup> phenotype was a consequence of the disruptive integration we analyzed 94 progeny from a cross between one of the transformant CR<sup>-</sup> strains with a *benA*<sup>+</sup>, *pyrG*<sup>-</sup> strain, GB13, and found that 52 of the progeny were *pyrG*<sup>-</sup>, CR<sup>+</sup> (conidiation sensitive); 41 were *pyrG*<sup>+</sup>, CR<sup>-</sup>; and 1 was *pyrG*<sup>+</sup>, CR<sup>+</sup>, which indicates that the CR<sup>-</sup> phenotype

co-segregated with the integrated *pyr4* marker. The one segregant that was both CR<sup>+</sup> and *pyrG*<sup>+</sup> may have been a recombinant or a case where the integrated marker excised and reintegrated at another locus. These data support the conclusion that the beta3-tubulin is the product of the beta14 gene.

## DISCUSSION

Two beta-tubulin genes, beta5 and beta14, have been cloned from *Aspergillus nidulans*. The beta5 and beta14 genes have different restriction maps and do not cross-hybridize in genomic blots when washed at high stringency. Thus, although the two cloned genes both code for beta-tubulins, there has been substantial divergence between the two sequences. Divergence of beta-tubulin genes has been previously reported in both humans and *Drosophila* (3, 18).

Each of the beta-tubulin clones was used to construct integrative transforming plasmids carrying the *pyr4* gene of *Neurospora crassa*. The plasmids AlpGM1 (beta14 sequence) and AlpGM4 (beta5 sequence) complemented a *pyrG*<sup>-</sup> mutant of *Aspergillus*. Analysis of genomic DNA from transformants has shown that in 80% of the transformants AlpGM1 and AlpGM4 integrated into the homologous beta-tubulin sequence. One possible reason for this high frequency of homologous recombination may be the lack of sequence homology between the *pyr4* of *Neurospora* and the *pyrG* of *Aspergillus*, thus eliminating the *pyrG* gene as a potential landing site for the plasmids.

Transformants that have the *pyr4* gene integrated at either the beta5 or beta14 gene have been crossed with a *pyrG*<sup>-</sup>, *benA*<sup>+</sup> strain to test for their linkage to *benA*. The beta5 clone was shown to be tightly linked to *benA*, which suggests its identity with *benA*. That the beta5 clone is *benA* was also demonstrated by its ability to suppress the temperature sensitive phenotype of *benA33*. In contrast the beta14-bearing plasmid showed little or no linkage to *benA*.

That only a single beta-tubulin gene was identified genetically, yet two genes could be cloned, raised some interesting questions. Why was only one gene identified using the mutational analysis, and what is the function of the second beta-tubulin gene? *Aspergillus* has at least seven different identifiable cell types: the vegetative mycelium, asexual spores (conidia), sexual spores (ascospores), hulle cells, conidiophores, and primary and secondary sterigmata. It is possible that a second beta-tubulin gene might have been missed if it functioned in a cell type other than the vegetative mycelium. In addition heterozygous diploids for *benA* are sensitive to benomyl and will no longer have a temperature-sensitive phenotype for *ts*<sup>-</sup>; *benA* alleles (reference 12 and this study). This immediately suggests an answer to the second question. The product of the beta14 gene is not required for vegetative growth. However, although vegetative growth in *benA* mutants is normal in the presence of benomyl, conidiation is blocked (27). It was reasonable therefore to expect that the beta14 gene product participated in conidiation and may be one of the many genes known to affect conidial development (4). We tested this possibility by using integrative recombination to disrupt the beta14 gene, and we showed that disruption of the beta14 gene in a *benA22* (benomyl resistant, GB20) background ablated the beta3 polypeptide and allowed conidia to develop in the presence of benomyl. Thus the beta14 (*tubC*) gene must be the structural gene for the beta3 polypeptide and we can therefore say that the *tubC* gene product,

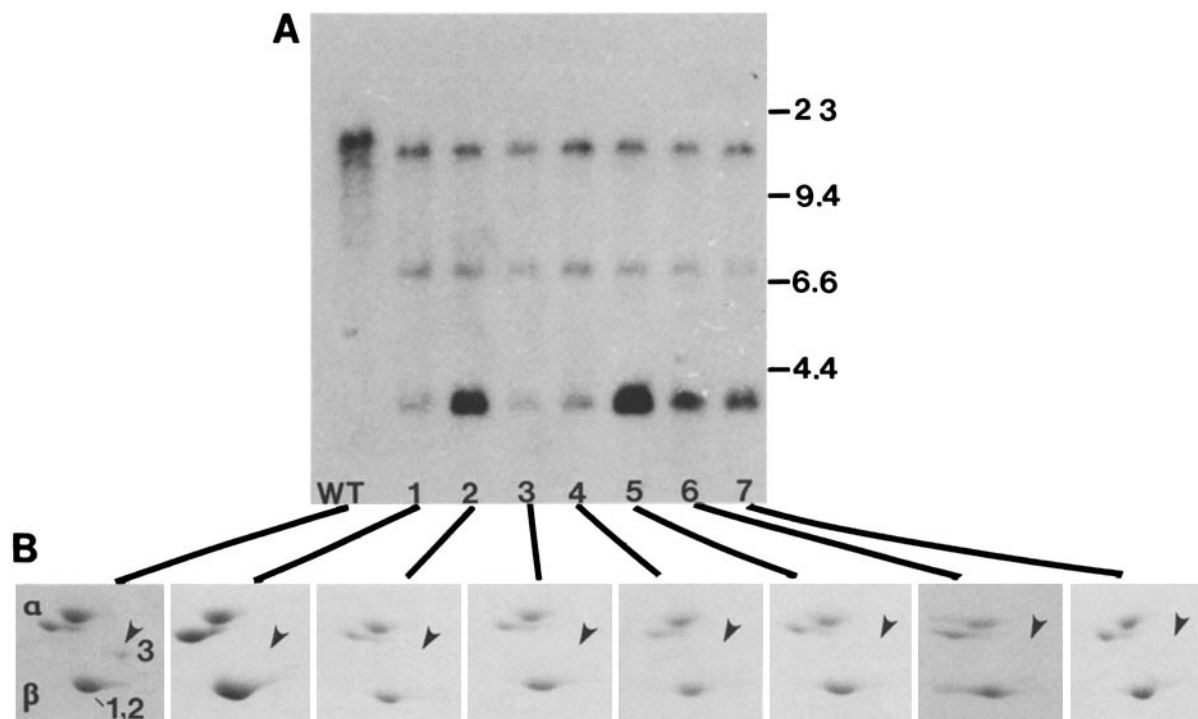


FIGURE 7 (A) Southern analysis of seven of the *AlpGM6* transformants that exhibited the conidial revertant phenotype and (B) two-dimensional gels of the tubulins of the same strains. Transformant DNA (2  $\mu$ g) was digested with *EcoR* I, fractionated on an agarose gel and transferred to nitrocellulose, and the filter was hybridized with a nick-translated insert from pB-14. Lane WT was total DNA from the nontransformed wild-type parental strain, and lanes 1–7 contain total DNA from the transformants. The position and size of lambda-DNA markers are indicated at the right. A tubulin-enriched fraction was prepared from the parental strain and the seven transformants. These fractions were run on two-dimensional gels (24). Only the tubulin region of each gel is shown, and the position of the beta3 polypeptide is marked with an arrowhead in each gel.

beta3, participates in conidial development. However, although the beta3 polypeptide participates in conidiation it is not necessary for the process in that conidiation proceeds in the absence of the beta3 polypeptide. Thus the beta1- and beta2-tubulin of *benA* can substitute for beta3-tubulin in conidiation.

How might the absence of the beta3-tubulin allow conidiation in the presence of benomyl? The results presented above suggest two general models. The first model suggests that beta3 participates in conidial development by forming microtubules that are a co-polymer of beta1- and/or beta2- and beta3-tubulins. This co-polymer would form benomyl-sensitive microtubules by incorporation of the beta3 subunit. The second model is based on a switch of beta-tubulin subunits such that the beta3 replaces the beta1 and beta2 subunits. This model predicts that the beta1 and beta2 subunits are made during vegetative growth and are repressed during conidiation. Either model would result in the presence of benomyl-sensitive microtubules during conidiation.

Both models make certain predictions. First, beta3-tubulin will be found in increased quantities during conidiation. This requires that *tubC*, the structural gene for beta3, be developmentally regulated. That a small quantity of beta3 is detected in vegetative cultures might argue against this possibility. However, this small quantity probably represents either a constitutive amount of beta3 synthesis produced during vegetative growth, or a contamination of the vegetative culture from a portion of the culture that sticks to the wall of the culture flask and initiates conidial development. Either possibility could explain the presence of the small amount of

beta3 observed in vegetative cultures. A second prediction is that beta3-containing microtubules should be found only in cells unique to conidial development. In these cells beta3 may be found as a co-polymer with beta1 and/or beta2 or it could be found as a homopolymer without these beta-tubulin. We are examining these possibilities.

The levels at which the synthesis and function of the beta3 polypeptide might be regulated are numerous. These include the synthesis and degradation of the mRNA at the transcriptional or posttranscriptional level, and the synthesis of the protein and/or its processing or its incorporation into a unique class of microtubules. Clearly, the low abundance of the beta3 polypeptide in vegetative cultures suggests that its synthesis is regulated or it is found in a unique cell type that is in low abundance. Normal vegetative growth of *benA*<sup>r</sup> (benomyl resistant) strains and their inability to conidiate in the presence of benomyl argues against the synthesis of beta3 in vegetative cells. In contrast, it is presumably the presence of beta3 in conidial-forming cells that makes conidiation sensitive to benomyl in *benA*<sup>r</sup> strains. This suggests that beta3 is made during conidial development and incorporated into microtubules. These microtubules may or may not contain the beta1 and beta2 polypeptides. In either case benomyl sensitive microtubules would be the result. There is good evidence for developmental specific beta-tubulin polypeptides in *Drosophila melanogaster* and *Physarum* (2, 16, 19).

This raises the question, Why does *Aspergillus* have more than one beta-tubulin gene? This is especially interesting in light of the present study, where the evidence indicates that the *tubC* gene product, beta3, can be substituted for by the

*benA* gene products, beta1 and beta2. This question has arisen in the past and was used to formulate the multitubulin hypothesis (5). One explanation is that the various tubulin species have evolved from a prototypical gene to perform specific functions. Alternatively, different tubulin genes have arisen to allow for independent control of their expression and presumably function. Either or both of these explanations could apply here. We are currently determining at what level(s) the *tubC* and *benA* genes are regulated in conidial development as well as in vegetative growth. In addition, it will be interesting to examine the types of microtubule structures formed during conidiation and the function of these structures. For example, Is the beta3 polypeptide found in a unique microtubule structure? Is it possible that the *benA* gene products, beta1 and beta2, are also found in cells associated with conidial development, but not as a co-polymer with beta3, or do they form co-polymers? All of these possibilities will have to be investigated before we can arrive at a full understanding of the functional relationships among the tubulin genes of *A. nidulans*.

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