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Abstract

The isolation of the beta-tubulin gene from a benomyl-resistant *Neurospora crassa* strain (Orbach et al. 1986 Mol. Cell. Biol. 6:2452-2461) has provided a dominant selectable marker usable in transformation experiments with *N. crassa*.

Mitotic instability in benomyl-resistant transformants of a fluffy strain of *Neurospora crassa*

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The isolation of the beta-tubulin gene from a benomyl-resistant *Neurospora crassa* strain (Orbach et al. 1986 Mol. Cell. Biol. 6:2452-2461) has provided a dominant selectable marker usable in transformation experiments with *N. crassa*.

In order to determine whether plasmid pBT6 (bearing the benomyl-resistant beta-tubulin gene on the pUC12 vector) stably integrated into genomic DNA, we used it to transform the morphological mutant fluffy of *N. crassa* (FGSC 45). This strain can be conveniently used in transformation experiments because it generates only uninucleate microconidia, allowing the isolation of homokaryons and avoiding the complications in isolation via ascospores (Rossier et al. 1985 Curr. Genet. 10:313-320). The fate of pBT6 DNA was analyzed in transformants grown under selective or non-selective conditions.

To produce microconidia, the fluffy strain was grown for 7 days at 25 C in Petri plates on Westergaard and Mitchell's synthetic medium supplemented with 1% acetate as sole carbon source (acetate medium). Transformation experiments were performed according to Orbach et al. (1986 Mol. Cell. Biol. 6:2452-2461) with modifications. Microconidia were inoculated in Neurospora minimal medium (Difco no. 0817-01) at a concentration of 1 x 10(7) cells per ml. Cultures were incubated 24 hours at 33 C at 150 rpm. Germinating microconidia (about 5 x 10(8)) were washed 3 times with distilled water at room temperature and resuspended in 10 ml 1 M sorbitol to which 2 ml Novozym 234 (Novo Industri A/S, batch 1199, 5 mg/ml in 1 M sorbitol) were added. Digestion was performed for 1 hour at 30 C at 100 rpm. After centrifugation, spheroplasts were washed twice with 10 ml 1 M sorbitol and once with 10 ml 5 mM Tris-HCl buffer pH 8.0, 1 M sorbitol, 50 mM $CaCl_2$ (transformation buffer). Spheroplasts in transformation buffer were diluted with 0.6 volume distilled water just before transformation (Akins and Lambowitz 1985 Mol. Cell. Biol. 5:2272-2278). Five microliters of DMSO and 0.1 ml 50 mM Tris-HCl pH 8.0 containing 40% polyethylene glycol 4000 (PEG) (BDH) and 50 mM $CaCl_2$ were then added to the spheroplasts in 0.4 ml diluted transformation buffer. Ten micrograms of plasmid pBT6 DNA purified twice by CsCl ultracentrifugation in 100 ul TE, pH 8.0, and preincubated 10 min with 25 ul heparin solution (5mg/ml, freshly prepared in transformation buffer), were then added to the transformation mixture. After 30 min incubation on ice, 5 ml PEG-Tris-$CaCl_2$ solution were added and incubation continued for 20 min at room temperature. Appropriate aliquots of the transformation mixture were inoculated with 3% agar, 1 M sorbitol, 2% sorbose, 0.05% fructose and 0.05% glucose) at 45 C. This medium was poured on a 25 ml bottom layer (Vogel's medium N containing 2% sorbose, 0.05% fructose, 0.05%

glucose and 1.5% agar to which 0.5 ug/ml benomyl was added after autoclaving). Plates were incubated for 3 days at 33 C.

Spheroplasts derived from microconidia germinated for 24 hours could be transformed with pBT6 with a frequency of 1000 to 4000 transformants per ug DNA. Microconidia incubated for 12 h were still ungerminated and were not suited for transformation due to poor formation of spheroplasts. Homokaryotic derivatives from the presumably heterokaryotic primary transformants were obtained by isolating colonies grown from microconidia inoculated on selective (0.25 ug/ml benomyl) Vogel's medium N supplemented with 2% sorbose, 0.05% fructose and 0.05% glucose.

The proportion of transformed nuclei and their stability in benomyl-resistant transformants were determined by genetic analysis of microconidia produced on selective (0.5 ug/ml benomyl) or non-selective acetate medium, assuming that the microconidia contain a random sample of the nuclei present in the mycelium. The percentage of transformed microconidia was determined by counting the colonies obtained from an equal number of microconidia plated onto selective (0.25 ug/ml benomyl) and non-selective Vogel's medium N (both contained 2% agar plus 2% sorbose, 0.05% glucose and 0.05% fructose). A variable degree of mitotic instability was observed during growth in the absence of selective pressure. Under such growth conditions, the microconidia of some transformants kept the transformed character (35% of benomyl resistant microconidia in transformant 1f) while microconidia of others lost it (<0.3% of transformed microconidia in transformant 3i).

To investigate at the molecular level the effects of the suppression of selective pressure on the fate of the transforming DNA, 3 transformants (1f, 9 and 3i) were grown in Vogel's medium N plus (0.25 ug/ml) or minus benomyl. DNA was then extracted and purified by CsCl ultracentrifugation. Undigested plasmid pBT6 DNA and undigested genomic DNAs from the transformants grown in the presence of benomyl were electrophoresed on 0.8% agarose gels and probed with nick-translated pBT6. No bands were detected that would correspond to the relaxed circular or supercoiled configurations of the plasmid (results not shown). This is consistent with the integration of pBT6 into genomic DNA and with the absence of free, autonomously replicating plasmids.

*Cla*I does not cut pBT6. In untransformed fluffy, pBT6 hybridized to a single *Cla*I DNA fragment of about 13 kb bearing the resident beta-tubulin gene (Fig. 1). In all genomic DNAs from the transformants, pBT6 hybridized to this band and to another *Cla*I fragment of higher molecular weight indicating that integration occurred by non-homologous recombination. However, the *Cla*I fragments bearing pBT6 sequences were shorter than the corresponding undigested DNAs, ruling out the presence of non-integrated circular oligomers of pBT6 (date not shown). In the absence of selective pressure, pBT6 sequences were maintained (transformant 1f) or lost (transformant 3i). In transformant 9, the intensity on the autoradiogram of the *Cla*I fragment bearing the pBT6 sequences is weaker after growth in the absence of selective pressure (Fig. 1). This suggests that some nuclei lost the pBT6 sequences, in agreement with the finding that only 7% of transformant 9 microconidia kept the transformed character under such growth conditions, as determined by genetic analysis.

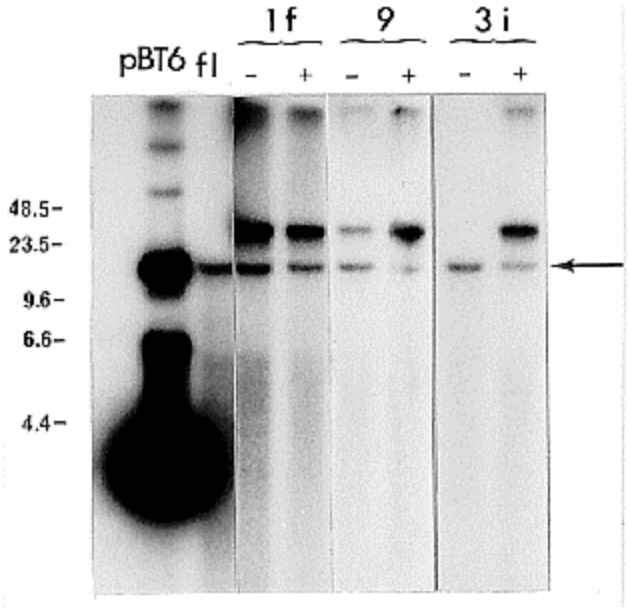

Figure 1. Genomic Southern blot analysis of DNAs isolated from untransformed strain fluffy (*fl*) of *N. crassa* and from 3 benomyl-resistant transformants (1f, 9 and 3i). Transformants were grown in the absence of selective pressure (-) or in the presence (+) of 0.25 µg/ml benomyl. 1 ug of DNA from each strain and 1 ng of plasmid pBT6 were electrophoresed after digestion with *Cla*I on 0.8% agarose gels and probed with 32P-labelled pBT6. As *Cla*I does not cut pBT6, it migrated mainly in the supercoiled configuration. The arrow points to the 13 kb *Cla*I DNA fragment bearing the resident beta-tubulin gene. Size markers (lambda DNA digested with *Hin*dIII) are shown in kb; *fl*: untransformed fluffy strain.

These results strongly suggest that mitotic instability observed in the absence of selective pressure is due to excision of integrated plasmid sequences. These observations contrast with the mitotic stability observed in *N. crassa* with other transformation systems (Case 1986 Genetics 113:569-587; Avalos et al. 1989 Curr. Genet. 16:369-372) and with the finding that pBT6 gave rise to mitotically stable transformants in *Podospora anserina* (Fernandez-Larrea and Stahl 1989 Curr. Genet. 16:57-60).

Introduction of DNA into *N. crassa* can lead to sequence instability in the sexual phase of the life cycle (RIP process, Selker et al. 1987 Cell 51:741-752). Our results suggest that another mechanism operating during vegetative growth serves to preserve the organization of the *N. crassa* genome.

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