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Transformation of the Dermatophyte *Trichophyton mentagrophytes* to Hygromycin B Resistance

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A transformation system for the ringworm-producing dermatophyte *Trichophyton mentagrophytes* has been developed. The system employs the plasmid pH1S, which contains a bacterial hygromycin B phosphotransferase gene linked to *Cochliobolus heterostrophus* regulatory sequences (B. G. Turgeon, R. C. Garber, and O. C. Yoder, Mol. Cell. Biol. 7:3297-3305, 1987). This plasmid confers hygromycin B resistance to *T. mentagrophytes*. The DNA was stably integrated into the fungal genome, and the number and sites of integrations varied among transformants. Transformant clones were capable of infecting guinea pigs. This system opens the way for the molecular genetic analysis of the interaction of *T. mentagrophytes* with epithelial animal tissues.

Hygromycin B is an aminocyclitol antibiotic which specifically inhibits protein synthesis in both procaryotes and eucaryotes by interfering with translocation (2) and also by causing misreading (14). Hygromycin B resistance genes have been cloned and sequenced from *Escherichia coli* (3, 7, 11) and *Streptomyces hygroscopicus* (9, 19). Both resistance genes code for hygromycin B phosphotransferases which inactivate the antibiotic by phosphorylation (9, 11). By using the *E. coli hygB* gene, cloning vectors have been created for a variety of organisms ranging from *E. coli* to mouse cells (1, 3, 6, 8, 13, 16, 17). In particular, filamentous fungi transformation systems based on expression of the *hygB* marker have been intensively sought (for a review, see reference 4). The main subject of our work is to elucidate the structure and regulation of genes involved in the zoopathogenic and saprophytic growth of the ringworm-producing dermatophyte *Trichophyton mentagrophytes*. Molecular analysis of these genes requires the development of a transformation system in this species. Here we report the establishment of a reproducible DNA transformation protocol for *T. mentagrophytes* based on resistance to hygromycin B.

The wild-type strain *T. mentagrophytes* CECT 2793 (CBS 570.80) was used for all transformation experiments. Sabouraud dextrose medium (SD; Merck & Co., Inc.) was used for routine growth. The vector pH1S is a pBR322 derivative carrying the *E. coli hygB* gene fused to an 838-base-pair fragment of *Cochliobolus heterostrophus* DNA that acts as a promoter in this fungal organism (12, 15). All DNA manipulations were carried out as described previously (10).

Growth inhibition of wild type by hygromycin B. Growth inhibition of *T. mentagrophytes* was tested by conidial inoculation onto SD agar plates (SD plus 2% [wt/vol] agar) containing a range of concentrations of hygromycin B. Growth was completely inhibited at 20 μg of hygromycin B per ml. The frequency of spontaneous resistance to the antibiotic at a concentration of 100 μg/ml (the level used in the selective medium for transformation experiments; see above) was less than 5×10^{-8} for protoplasts. Thus, a selection procedure based on the acquisition of hygromycin B resistance seemed feasible for *T. mentagrophytes*.

Transformation of *T. mentagrophytes*. Protoplasts were produced with 1 mg of Novozym 234 (Novo Industri) per ml in 50 mM phosphate buffer (pH 5.8)–0.7 M KCl. They were washed with ST buffer (1.2 M sorbitol, 10 mM Tris hydrochloride, pH 8.0) and then with STC buffer (1.2 M sorbitol, 10 mM Tris hydrochloride, pH 8.0, 10 mM $CaCl_2$). In a typical experiment 10^8 protoplasts in 200 μl of STC buffer were incubated at 4°C for 20 min with 10 μg of plasmid DNA and 50 μl of PCT (60% [wt/vol] PEG 6000 in 10 mM $CaCl_2$–10 mM Tris hydrochloride, pH 7.5). A total of 1 ml of PCT was then added. After an additional 5-min incubation at room temperature, the protoplast suspension was centrifuged at 6,500 × *g* for 1 min, and the pellet was suspended in 200 μl of STC buffer. Aliquots of this protoplast suspension were mixed with 5 ml of RSD protoplast medium (SD plus 1.2 M sorbitol–2% [wt/vol] agar) and poured over RSD plates. To select for hygromycin B-resistant transformants, these plates were further overlaid 20 h later with 5 ml of RSD medium containing enough hygromycin B to give a final concentration of 100 μg/ml in the whole plate. All media were incubated at 28°C in the dark.

After 7 days, hygromycin B-resistant colonies were clearly discernible. These colonies appeared at a rate of 0.004 to 6 per μg of pH1S and per 10^6 viable protoplasts. No such colonies were detected when DNA-lacking controls were plated on selective medium. In addition to the resistant colonies, 3 to 5 days later a greater number of small colonies arose both on plus and minus pH1S plates. These colonies did not maintain a hygromycin B-resistant phenotype when transferred onto fresh medium containing 50 μg of hygromycin B per ml. These facts strongly suggest that these late colonies are not abortive transformants. We can speculate that this residual growth was due to a partial inactivation of the drug in the selective transformation medium.

Molecular analysis of hygromycin B-resistant colonies. For Southern hybridization analysis, a 1.58-kilobase *Eco*RI-*Eco*RI fragment from pH1S containing the hygromycin B resistance gene was used (15). Undigested DNA from two independent hygromycin B-resistant colonies (T36 and T38) showed a hybridization signal corresponding to the randomly sheared DNA at the top of the gel (Fig. 1, lanes c and g). Compared with this, no signal was detected when DNA

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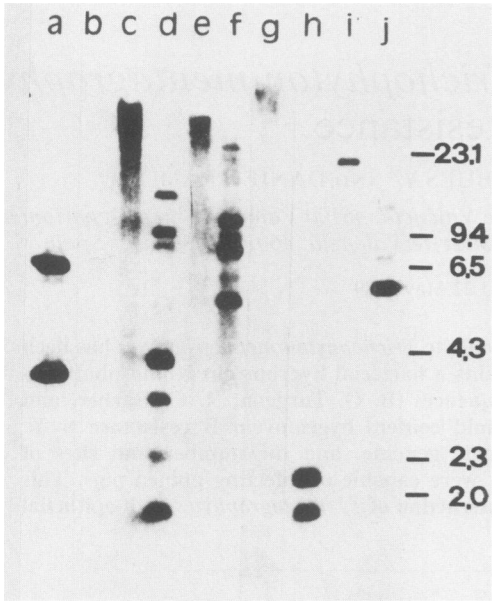

FIG. 1. Autoradiograph of DNA (10 μg per lane) from wild-type *T. mentagrophytes* and two independent transformants (T36 and T38) probed with the ^{32}P-labeled 1.58-kb *Eco*RI-*Eco*RI fragment from pH1S containing the *hygB* gene. Lanes: a, undigested pH1S (1 ng); b, undigested DNA from wild-type *T. mentagrophytes*; c, undigested T36 DNA; d, T36 DNA digested with *Eco*RI; e, T36 DNA digested with *Bam*HI; f, T36 DNA digested with *Sal*I; g, undigested T38 DNA; h, T38 DNA digested with *Eco*RI; i, T38 DNA digested with *Bam*HI; j, T38 DNA digested with *Sal*I. Sizes are indicated in kilobases at the right of the figure.

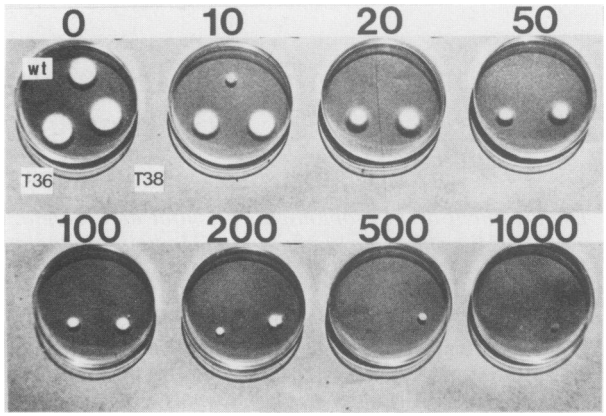

FIG. 2. Sensitivities of wild-type (wt) *T. mentagrophytes* and two independent transformants (T36 and T38) to various concentrations of hygromycin B. The number above each plate indicates the concentration of hygromycin B in micrograms per milliliter. Plates were incubated at 28°C for 6 days in the dark. Locations of colonies on each plate were as indicated for the top left plate.

from the untransformed wild-type strain was probed in this way (Fig. 1, lane b). This indicated, similarly to data from other filamentous fungi (5), that the transforming DNA was incorporated into the recipient genome. For a further investigation, we have analyzed DNA from T36 and T38 by Southern hybridization by using digestions with *Bam*HI (which does not cut pH1S), *Sal*I (which cuts pH1S once), and *Eco*RI (which cuts pH1S twice). The *Bam*HI analysis of both transformants showed that hybridization occurred in a single high-molecular-weight band (Fig. 1, lanes e and i). Since there is an absence of *Bam*HI restriction sites in pH1S, this result suggests that vector integration has been in a tandemly repeated way. The hybridization pattern of the transformant T38 for *Eco*RI and *Sal*I digestions was compatible with a tandem integration of two copies of the plasmid by the 1.58-kilobase *Eco*RI-*Eco*RI fragment of pH1S (Fig. 1, lanes h and j). The transformant T36 had a complex pattern (Fig. 1, lanes d and f) which reflects multiple sites of integration, or even sequence rearrangements within the individual copies in tandem, as in the case of the *amdS* gene in *Aspergillus nidulans* (18). Dot blot experiments revealed that the copy number of the *hygB* gene of the transformant T36 was at least twice that of the transformant T38. Thus, in T36, there must be at least four copies of the *hygB* gene.

Hybridization analysis of other transformants (data not shown) was in accordance with different random tandem integrations.

Hygromycin B resistance levels and mitotic stability of transformants. To determine the level of HmB resistance, T36 and T38 were grown in SD containing different concentrations of hygromycin B (Fig. 2). Both transformants had a more than 20-fold greater resistance to hygromycin B than did the recipient wild-type strain. The level of resistance of transformants (200 μg of hygromycin B per ml for T36 and 1,000 μg of hygromycin B per ml for T38) did not correspond directly to the number of *hygB* gene copies (T36 has at least twice as many copies as T38). These facts probably reflect a difference in the efficiency of the expression of the *hygB* gene.

Transformants were examined for mitotic stability of the transforming DNA. Purified single colonies of each transformant obtained through microconidia were grown and subcultured a total of five times on plates of nonselective medium (SD minus hygromycin B). The resulting colonies were transferred onto plates of selective (SD plus 50 μg of hygromycin B per ml) and nonselective media to test sensitivity to hygromycin B. For each sample, 3,000 colonies were scored, and all of them retained the hygromycin resistance phenotype. Southern analysis of DNA obtained from nonselective medium colonies corresponding to each transformant revealed the same hybridization pattern as their parental clones.

Virulence of hygromycin B-resistant transformants. T36 and T38 transformants were analyzed for virulence towards guinea pigs. A microconidial suspension in honey of each transformant was topically inoculated to the abraded skin of these animals. After 15 days, all of them showed ringworm lesions similar to those observed in control experiments with the wild-type strain. Transformants were then reisolated and tested for resistance to hygromycin B and for the presence of pH1S in their genomes. In both cases (T36 and T38), isolates recovered from infected tissue were hygromycin B resistant. The genomic bands homologous to the *hygB* gene observed before infection were also found after recovery from the infected animals. This fact strongly suggests that in these transformants, the plasmid was integrated at loci which are not involved in virulence and that the presence of foreign DNA in their genomes had no effect on the pathogenic process.

The results described here establish for the first time a DNA-mediated transformation system for the dermatophytic fungus *T. mentagrophytes*. The pH1S vector contains a *hygB* gene fused to a putative promoter of *C. heterostrophus*. This plasmid transforms, in a random integrative way,

to *T. mentagrophytes*. Transformant colonies were mitotically stable and could infect guinea pigs. We think that the low frequency of transformation can be explained if the putative *C. heterostrophus* promoter is not functional in *T. mentagrophytes*. This fact is in accordance with the similarly low frequencies obtained in *C. heterostrophus* with the truncated *hygB* gene without promoter (0.02 to 0.04 transformants per μg of DNA and per 10^6 viable protoplasts). Furthermore, the T38 transformant has fewer *hygB* gene copies but is more resistant to hygromycin B than is T36. This could be explained by an insertional fusion of an endogenous *T. mentagrophytes* promoter upstream of the *hygB* structural gene in T38. Experiments to test this possibility are in progress. The cloning of *T. mentagrophytes* promoters could open the possibility of constructing genomic fusions with the *hygB* gene, which would increase the transformation frequencies.

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