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Use of a bacterial Hygromycin B resistance gene as a dominant selectable marker in Neurospora crassa transformation.

Dominant transformation markers allow maximum flexibility in the choice of transformation recipients. The most widely used transformation marker in N. crassa has been the Neurospora gene conferring benomyl resistance (Orbach, et al. 1986 Mol. Cell. Biol. 6:2456-2461). Unfortunately, this marker is usually inactivated during sexual crosses, presumably because its introduction by transformation generates strains with duplicate ß-tubulin sequences that are subject to inactivation during the sexual cycle by RIP (Selker et al. 1987 Cell 51:741-752; Selker and Garrett, 1988 Proc. Natl. Acad. Sci. USA 85:6870-6874). We therefore looked for a dominant drug resistance marker that would not be homologous to any sequence in the Neurospora genome. A number of researchers have found that a bacterial gene encoding hygromycin B resistance (hph, hygromycin B phosphotransferase), if driven by a eukaryotic promoter, can confer drug resistance on eukaryotic cells. Hygromycin B (hygB) is an amino-glycoside that inhibits protein synthesis by causing mistranslation (Gonzalez et al. 1978 Biochim. Biophys. Acta 521:459-469) and by interfering with protein translocation (Singh et al. 1979 Nature 277:146-148). In fungi, Grits and Davies have had promising results in the use of the hph gene in yeast (Gritz and Davies 1983 Gene 29:179-188). Punt et al. and Cullen et al. have had success using this gene in both A. nidulans and A. niger (Punt et al. 1987 Gene 56:117-124; Cullen et al. 1987 Gene 57:21-26). Here we report the sensitivity of Neurospora to hygB and the use of two existing plasmids and derivatives as transformation markers in Neurospora.

All of the strains of Neurospora that we have tested (approx. 15) are sensitive to hygB. We generally use Vogel's minimal medium with sorbose for plates or sucrose for slants. We have selected resistance in a number of supplemented media, including a complete medium. HygB was obtained from Calbiochem. The commercial powder is dissolved in sterile water at 200 mg/ml and added to autoclaved media. Media can be stored for several months at 4°C. A number of different sensitivity tests have been performed. In general, strains are sensitive to 150 ug/ml hygB. Several different plating conditions have been used to isolate transformants produced by standard procedures (Vollmer and Yanofsky 1986 Proc. Natl. Acad. Sci. USA 83:4869-4873). Our standard conditions involve plating 5 x 10^6 spheroplasts on a 25 ml sorbose plate (200 ug/ml hygB) in 8 ml top agar lacking hygB. Top and bottom layers usually contain 1.5% agar. Decreasing the volume of top agar or increasing the hygB concentration increases the stringency of the selection and improves the background, but discriminates against some transformants and lowers the apparent transformation frequency. We have used similar selection conditions to isolate hyg^r mutants from mutagenized conidia. The frequency of spontaneous mutation to hyg^r is not high enough to interfere with the selection.

Our initial transformations used either pAN7-1 (Punt et al. 1987 Gene 56:117-124) or pDH25 (Cullen et al. 1987 Gene 57:21-26). These plasmids contain Aspergillus transcription signals that direct the expression of the E. coli hph gene. We have made derivatives of these plasmids that other researchers may find useful. Two of these, pCSN43 and pCSN44, contain a SalI fragment of pDH25 that confers resistance as well as pDH25. These plasmids provide additional unique cloning sites (Figure 1A, 1B). They offer the additional advantage that they replicate to a very high copy number in E. coli. The SalI fragment from pDH25 that is present in pCSN43 and pCSN44 has been transferred into several other plasmids, and has conferred hygB^r in each case. In pES200 the trpC terminator has been deleted from pDH25 leaving a single BamHI site (Figure 1C). This plasmid is as effective as pDH25 in conferring hygB resistance; the single BamHI site allows convenient cloning of BamHI compatible restriction fragments. We have not mapped the actual transcription signals or start sites that direct hph gene expression in N. crassa, though the structure-activity relationship for the trpC promoter has been studied in Aspergillus (Hamer and Timberlake 1987 Mol. Cell. Biol. 7:2352-2359). Surprisingly, insertion of a 430 bp TaqI fragment from the zeta region (Selker and Stevens, 1985 Proc. Natl. Acad. Sci. USA 82:8114-8118) into the ClaI site of pDH25, separating the promoter from the hph coding region, did not abolish hygB^r. However, the ClaI-BamHI fragment containing the hph coding region is not sufficient for resistance. Plasmids pES200, pCSN43 and pCSN44 are available from the Fungal Genetics Stock Center.

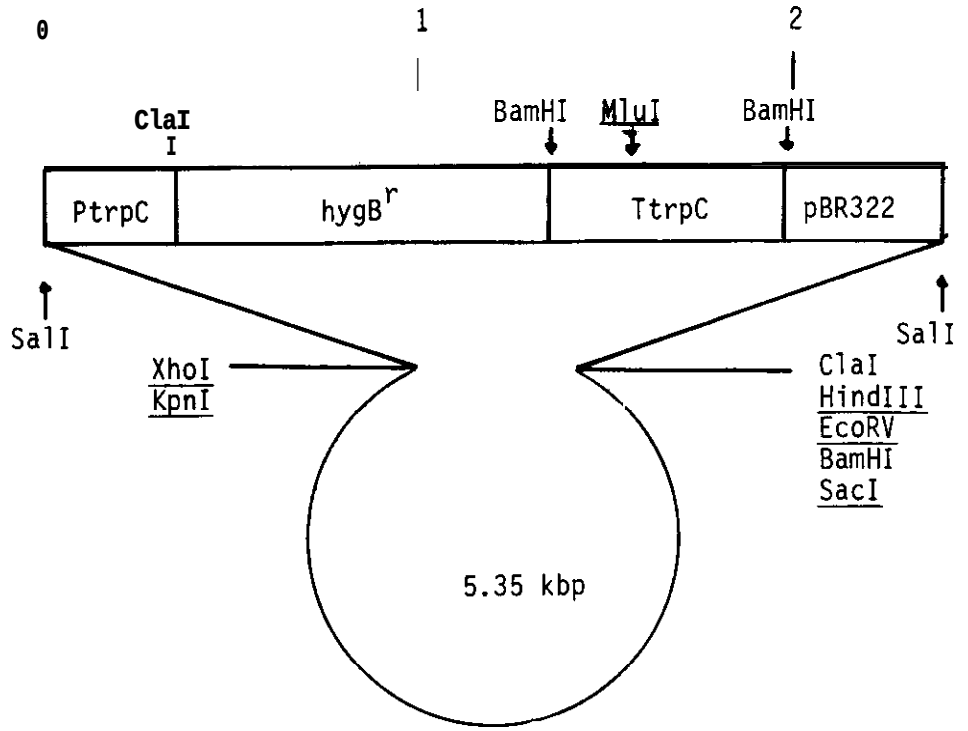

Figure 1A: pCSN43 contains the 2.4 kbp SalI site from pDH25 cloned into the SalI site of pBSSK^+ (Stratagene). The indicated scale is in kilobase pairs. Unique restriction sites are underlined. The promoter and terminator fragments from Aspergillus trpC are also indicated. Additional restriction sites in these plasmids may be unique. Those indicated have been tested.

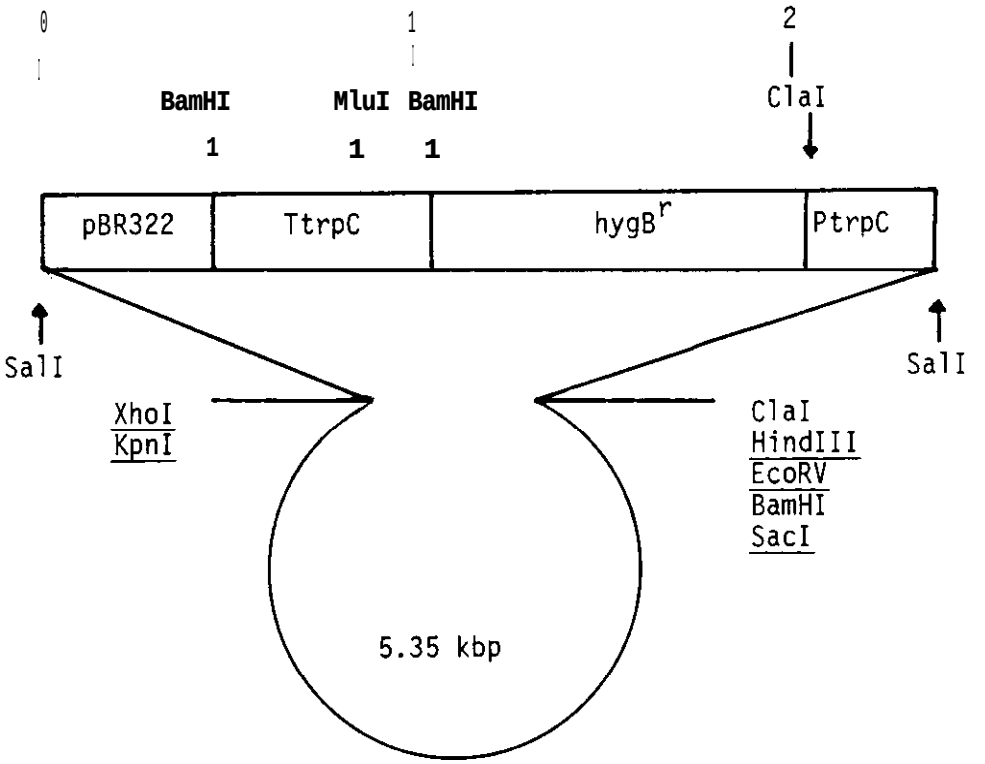

Figure 1B: pCSN44 has the SalI fragment from pDH25 oriented opposite to the orientation in pCSN43.

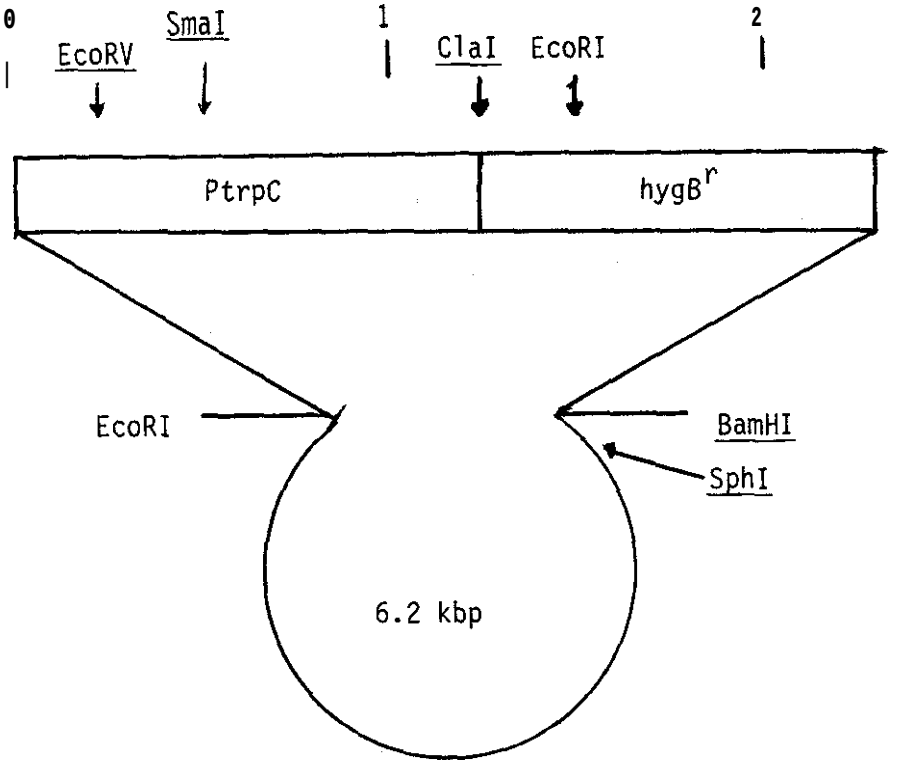

Figure 1C: pES200 was constructed by deleting the 710 bp BamHI fragment from pDH25. Sites discussed in the text are indicated. The position of the trpC promoter and hygB^r are indicated. Unique cloning sites are underlined. Vector sequences are represented by the thin circle; the resistance gene and its transcription signals are drawn to scale as a thick box.

Transformation frequencies with the hph gene are similar to those observed with the benomyl resistance gene. Mixtures of plasmids containing the two markers cotransform at frequencies up to 75%, suggesting that there is little difference between their transformation frequencies. We observe 1-10 x 10^3 transformants/ug DNA under the conditions that we have described. Transformants appear to fall into two classes: those resistant to 200 ug/ml and those resistant to >1 mg/ml. We have not determined the difference between classes. Southern hybridization of various transformants indicates that one to several copies of the gene have integrated, as observed for most other transforming DNAs. Transformants typically yield hygromycin resistant progeny in sexual crosses with wild type. The hph marker is inactivated when known multicopy transformants are crossed to wild type

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