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Hygromycin B Phosphotransferase as a Selectable Marker for DNA Transfer Experiments with Higher Eucaryotic Cells

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The DNA coding sequence for the hygromycin B phosphotransferase gene was placed under the control of the regulatory sequences of a cloned long terminal repeat of Moloney sarcoma virus. This construction allowed direct selection for hygromycin B resistance after transfection of eucaryotic cell lines not naturally resistant to this antibiotic, thus providing another dominant marker for DNA transfer in eucaryotic cells.

Hygromycin B (hmB) is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* (11) which inhibits protein synthesis in both procaryotes and eucaryotes by interfering with ribosomal translocation and with aminoacyl-tRNA recognition (2, 3, 12). A dominant gene conferring resistance to hmB would therefore provide another useful selectable marker in experiments involving gene transfer in and between procaryotic and eucaryotic cells. Recently a plasmid-coded hmB phosphotransferase (hph) was identified in *Escherichia coli*, and its complete nucleotide sequence was determined (5). This gene was successfully used as a selectable marker for transformation of *Saccharomyces cerevisiae* (5).

The sensitivity of various cultured cell lines to hmB was assessed by plating cells at low density in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and hmB to give final drug concentrations ranging between 50 and 400 μg/ml of medium. The medium plus drug was changed every 4 to 5 days. Usually one or two cycles of replication still occurred before the onset of cytotoxicity; cell death commenced ca. 3 days after the beginning of drug treatment and was generally complete after 8 days (CV1 and HeLa cells sometimes required 10 to 12 days before cell killing was complete). So far, no cell line has been found that is naturally resistant to hmB. Furthermore, the use of an ionophore to permeabilize the cell membrane concomitantly increased the sensitivity of HeLa cells to hmB (1). Several primary cell lines were also observed to be sensitive to hmB; among those were a *Xenopus* kidney tubule cell line and a rabbit mammary gland cell line. Other animal cell lines found to be sensitive to hmB included mouse cell lines LTK^-, C127, NIH 3T3, and GR; hamster cell line CHO; rat cell line HTC; mink cell line CCL64; cat cell line AZ171; and monkey cell lines TC7 and CV1. Hmb is also extremely cytotoxic to all species of *Leishmania* tested (J. Bouvier, personal communication).

To attempt expression of the *hph* gene in higher eucaryotic cells, we used pMLTR-I as a vector. pMLTR-I originates from pMLTR (16) from which one of the two Moloney sarcoma virus (MoSV) long terminal repeats (LTRs) was removed by digestion with *Sma*I. The MoSV LTR contains all of the regulatory sequences necessary for viral expression (15). The DNA coding sequence of the *hph* gene was excised as a *Bam*HI fragment from pLG88 (like pLG89) (5), blunt ended, and ligated into the *Sma*I site of pMLTR-I to yield pY3 (Fig. 1). Thus, the *hph* coding sequence is inserted 3′ to the MoSV enhancer elements, TATA box, cap site, and ribosome binding site and 5′ to the viral polyadenylation signal. This construction was then transfected into eucaryotic cell lines.

The day before transfection, LTK^- and CCL64 cells were plated at a cell density of 2×10^5 cells per 6-cm dish. pY3 plasmid DNA (~70% supercoiled) was coprecipitated with calcium phosphate along with 10 μg of carrier DNA (4). The precipitate was left on the cells for ca. 20 h, and selective medium containing 200 μg of hmB per ml for LTK^- cells and 400 μg of hmB per ml for CCL64 cells was added to the cells 48 h after transfection. Resistant clones were first observed after 7 to 9 days and were picked individually with cloning cylinders 14 to 21 days after the selection was applied. Transfection efficiencies were 25 to 40 clones per μg of pY3 per 2×10^5 LTK^- cells plated and ca. 5 clones per μg of pY3 per 2×10^5 CCL64 cells plated. HmB-resistant clones were also obtained from CV1 cells at an approximate efficiency of 1 clone per μg of pY3 per 10^5 CV1 cells plated (D. Cribbs, personal communication). Once established, the transfected LTK^- cells remained fully resistant to hmB even after ca. 100 cell generations of growth in the absence of selection. LTK^- cells converted to hmB resistance still retained their sensitivity to G418, another aminoglycoside antibiotic. Conversely, CHO cells transfected with the *neo* gene encoding a phosphotransferase which modifies G418 (13), although resistant to G418, remain sensitive to hmB (H. C. Hurst, personal communication). This therefore suggests well-defined substrate ranges for both of these aminoglycoside antibiotic phosphotransferases and opens the possibility of using both of these selection markers in sequential transfection experiments.

Total cellular RNA was extracted from hmB-resistant cell lines as previously described (9). Genomic DNA was extracted simultaneously through treatment of the pellet after centrifugation through sucrose with 0.05% sodium dodecyl sulfate and 0.1 mg of proteinase K per ml (6). The DNA was digested with *Kpn*I and *Sst*I, electrophoresed, transferred to nitrocellulose paper, and hybridized to the ^{32}P-labeled concatemerized *Bam*HI insert of pLG88 (10). The only band that was found to hybridize to the probe in the DNA of the hmB-resistant cell lines had a size of 1.08 kilobases, representing all of the *hph* coding sequence plus the adjoining MoSV sequences including the TATA box present on a *Kpn*I/*Sst*I restriction fragment (Fig. 2). Thus, there are no discernible rearrangements within that region of the DNA from transfected cells. No hybridization was detected to the genomic

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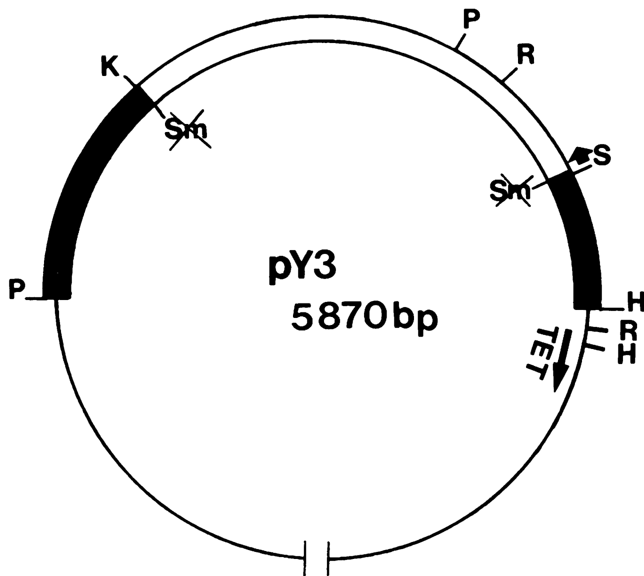

FIG. 1. Restriction map of pY3. The *Bam*HI insert of pLG88 was ligated into the *Sma*I site of pMLTR-I. Abbreviations: H, *Hin*dIII; K, *Kpn*I; R, *Eco*RI; Sm, *Sma*I; S, *Sst*I; P, *Pst*I. Symbols: ——, pBR322 sequences; ■, MoSV sequences; □, *Bam*HI insert of pLG88; arrow, site of initiation and direction of transcription.

DNA of untransfected LTK⁻ cells. All of the hmB-resistant cell lines examined appear to have integrated 1 to 20 copies of the *hph* gene per cell.

Polyadenylate-containing RNA was isolated by oligodeoxythymidylate-cellulose column chromatography, electrophoresed, transferred to nitrocellulose paper, and hybridized to the ^{32}P-labeled *Bam*HI insert of pLG88 (14). A band corresponding to the expected size of 1.2 kilobases was

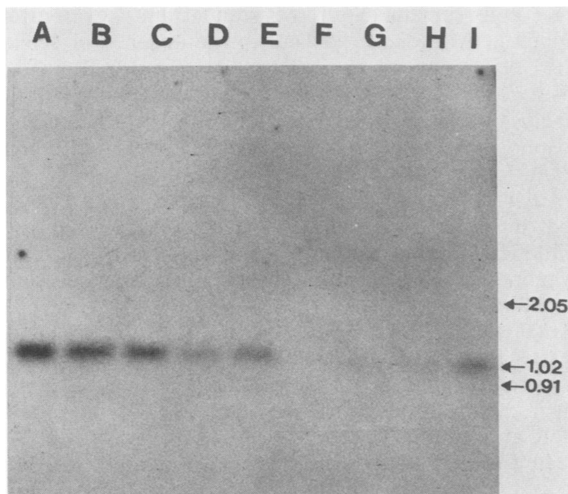

FIG. 2. Analysis of *hph*-specific sequence content and organization in genome DNA of LTK⁻ cell. Genomic DNA (10 μg) from pY3-transfected, hmB-resistant LTK⁻ cell (lanes A to E) and untransfected LTK⁻ cells (lane F) was digested with *Kpn*I and *Sst*I and hybridized to the ^{32}P-labeled, concatemerized *Bam*HI insert of pLG88. Lanes G to I, *Bam*HI inserts of pLG88 equivalent to 1, 2, and 5 copies per genome, respectively, of the *Kpn*I/*Sst*I fragment of pY3.

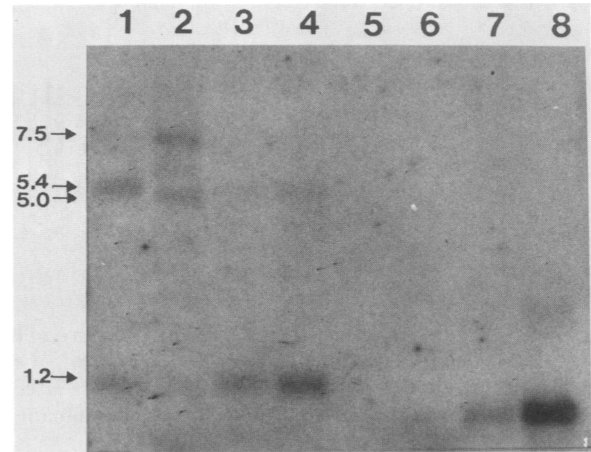

FIG. 3. Analysis of *hph*-specific mRNAs in LTK⁻ cells. Polyadenylate-containing RNA (5 μg) from pY3-transfected, hmB-resistant LTK⁻ cells (lanes 1 to 4) and untransfected LTK⁻ cells (lane 5) was hybridized to a ^{32}P-labeled *Bam*HI insert of pLG88. Lanes 6 to 8, *Bam*HI inserts of pLG88 equivalent to 2, 20, and 80 pg of *hph*-specific mRNA, respectively.

found to have hybridized to the probe in all lanes containing RNA from hmB-resistant cell lines (Fig. 3). No *hph*-specific mRNA was detected in untransfected cells. Assuming 0.15 pg of polyadenylate-containing RNA per cell (M. Carneiro, J. Mol. Biol., in press), the number of molecules of *hph* mRNA per LTK⁻ cell was calculated to range between 2 and 10. The exact origin of the larger mRNA species seen to hybridize is still unclear; they may result from transcription initiation upstream or polyadenylation downstream of the normal MoSV regulatory sequences or from both. When a *mos* hybrid gene was inserted into the *Sma*I site of pMLTR-I, transcription was found, by S1 mapping, to initiate correctly at the cap site in the viral LTR (F. A. van der Hoorn, manuscript in preparation). It is pertinent to assume that this also applies to the transcription of the *hph* gene in the same construction, at least for the synthesis of the 1.2-kilobase mRNA species.

It is interesting that the *hph* mRNA contains an additional AUG seven bases upstream from the protein-coding sequence, the in-phase termination codon of which would lie 380 bases beyond the presumptive *hph* translation initiator codon (12). Although initiation of translation of eucaryotic mRNAs typically occurs at the first AUG codon from the 5′ end of the message, several exceptions have been described. In fact, a similar observation has been made with the *neo* gene (13). Additional upstream AUG codons that are out of phase with the protein-coding sequence can severely reduce protein yields, depending on the flanking sequences (7, 8, 15, 16). However, removal of the first ATG from the DNA coding sequence of the *hph* gene actually appeared to lower the expression in *Saccharomyces cerevisiae* (5).

HmB is now commercially available from Calbiochem-Behring. This drug, along with the phosphotransferase gene that modifies it, provides a generally applicable selection system for DNA transfer experiments between both procaryotic and eucaryotic cells.

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