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Hygromycin Resistance as a Selectable Marker in *Dictyostelium discoideum*

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We have constructed an expression cartridge which has the bacterial hygromycin resistance gene (*hph*) fused to the *Dictyostelium discoideum* actin 15 promoter, with a segment of 3′-flanking DNA from the actin 15 locus placed downstream of the *hph* gene to serve as a transcription terminator. The plasmid pDE109, which contained this cartridge and a *Dictyostelium* origin of replication, transformed *D. discoideum* with high efficiency under hygromycin selection. The availability of this selectable marker circumvents the previous limitation of having G418 resistance as the only selectable marker for this organism; secondary transformation can now be used to introduce DNA into previously transformed cell lines.

The cellular slime mold *Dictyostelium discoideum* has been used for a wide variety of biological studies, including biochemical, developmental, and molecular genetic studies (10). The establishment of a DNA-mediated transformation system (8) has dramatically increased the types of experimental questions which can be addressed with this organism. The usefulness of this transformation system, however, is currently limited by the availability of only a single selectable marker which can be used to introduce DNA into the organism, resistance to the neomycin derivative G418. This limitation precludes many sorts of molecular genetic experiments, such as introduction of cloned genes into gene-disrupted lines or antisense lines. In these situations, the initial transformed line of interest is already resistant to G418 and thus cannot serve as a recipient in further transformations with that selectable marker. We report here that we have overcome this difficulty by constructing a vector that contains a hygromycin phosphotransferase gene (*hph*) and can be used to transform *D. discoideum* with the antibiotic hygromycin B used as the selective agent (3).

MATERIALS AND METHODS

Plasmid constructs. All DNA manipulations were done by standard procedures (6). The plasmid pUC119 (11) was first restricted with *Hin*dIII, filled in with Klenow enzyme, and then religated, yielding pUC119NH. The plasmid pSC79 contains the actin 15 gene of *Dictyostelium discoideum* on a 4.1-kilobase (kb) *Cla*I-*Bgl*II (5) fragment cloned into *Cla*I-*Bam*HI-restricted pBR322. This plasmid was restricted with *Sph*I, treated with T4 DNA polymerase to yield blunt ends, and then restricted with *Xba*I. This treatment liberated the actin promoter and coding region and 200 base pairs (bp) of downstream vector as a 2.2-kb fragment. pUC119NH was restricted with *Bam*HI, blunted with Klenow, and then restricted with *Xba*I. The 2.2-kb actin gene fragment was ligated into this vector to create pDE101. The blunted *Sph*I-*Bam*HI junction of pDE101 regenerates a *Bam*HI site. The hygromycin resistance gene (*hph*) was obtained from the plasmid pLG90. pLG90 is identical to pLG89 (3) except that the downstream *Bam*HI linker is located approximately 30 bp downstream of the *hph* gene stop codon instead of in the coding region. Digestion of pLG90 with *Bam*HI liberated the *hph* gene as a 1-kb fragment. This fragment was ligated into the *Bam*HI site of pUC119 to yield pUCHygR. (In this construct the *hph* gene lies in opposite orientation from the *lacZ* sequences of the vector.) pUCHygR was digested at the polylinker *Hin*dIII and *Sac*I sites, and the 1-kb *hph* gene fragment was isolated. This fragment was then restricted with *Hph*I and treated with T4 DNA polymerase to create blunt ends. This treatment created a blunt end inside the *hph* coding region, just after the third codon of the *hph* gene. This fragment was then restricted with *Xba*I, creating a sticky end in the polylinker 3′ to the coding region. pDE101 was next restricted with *Hin*dIII and treated with Klenow enzyme. This treatment yielded a blunt end at the eighth codon of the actin gene. This restricted plasmid was then restricted with *Spe*I, which cuts in the 3′-flanking sequences of the actin 15 gene. The *hph* gene fragment described above was ligated into the vector portion of the pDE101 digest, replacing the coding region of the actin 15 gene with the coding region of the hygromycin resistance gene. This step fused the actin promoter and the first eight codons of the actin 15 gene to the third codon of the hygromycin resistance gene. At the 3′ end of the *hph* gene in this construct there is a 265-bp segment from the 3′-flanking region of the actin 15 gene which serves as a terminator. This plasmid is named pDE102.

pNEO-MLS, a derivative of pA15TX (2), was made as follows. A modified version of pA15TX in which *Bam*HI and *Eco*RI linkers were inserted at *Pvu*II and *Sal*I sites, respectively (2), was restricted with *Bam*HI and *Xba*I, filled in with Klenow, and then religated. This treatment removed the intervening polylinker and recreated the *Bam*HI site. The actin 15 promoter-Neo^r cartridge of pNEO-MLS was liberated as a 2.2-kb *Bam*HI-*Bgl*II fragment, with the *Bgl*II site made blunt-ended with Klenow enzyme. This fragment was ligated into pDE102 which had been restricted with *Kpn*I (treated with T4 DNA polymerase to make it blunt ended) and *Bam*HI. The resultant plasmid, pDE104, has both Hyg^r and G418^r cartridges, both driven by actin 15 promoters and sharing an actin 15 terminator which lies between them. pDE109 was made by cloning the 5.9-kb Ddp2 sequence from pnDSal (9; B. Leiting and A. Noegel, Plasmid, in press) into the *Sal*I site of pDE102.

***Dictyostelium* transformations.** Transformations by calcium phosphate precipitates were carried out by minor modifications of previously described methods. Calcium phosphate-

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DNA precipitates were made as described by Nellen et al. (7), except that medium used during these treatments was buffered with 20 mM Bis-Tris instead of MES (morpholineethanesulfonic acid). Generally, 10 μg of DNA and 10^7 cells were used for each transformation. After the glycerol shock, cells were allowed to recover overnight (18 to 24 h) in Bis-Tris medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was then removed, and the cells were suspended in HL5 containing hygromycin or G418 plus penicillin and streptomycin. Stock solutions of hygromycin (Calbiochem, >98% pure) were made in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5), filter sterilized, and stored in portions at −20°C. Hygromycin was used at 40 μg/ml in initial experiments, although more recently we have found that selections at 25 to 35 μg/ml give better results. G418 was used at 10 μg/ml. For most transformations, cells were suspended in the selective medium and then transferred directly to 96-well microtiter plates. Generally, dilutions of the initial resuspension were made, and these were also plated in microtiter plates to ensure that one set of plates would give clonal colonies. Medium on transformation plates was changed every 3 to 5 days on high-density petri plate cultures and every 5 to 7 days on diluted microtiter plate cultures.

Electroporation was performed under the conditions described by Howard et al. (4), with some modifications. *Dictyostelium* cells grown in HL5 medium were washed twice in 10 mM sodium phosphate (pH 7.0)–50 mM sucrose and suspended in the same buffer at 10^7 cells per ml; 0.8 ml of this suspension was transferred to a Bio-Rad Gene Pulser cuvette, mixed with 5 μg of CsCl-banded plasmid, and then placed in an ice bucket for 10 min. The cuvette was then mixed briefly to resuspend cells, which were immediately electroporated with a Bio-Rad Gene Pulser at 1,200 V and 3 μF settings, giving a time constant of 0.6 to 1.0 ms. Substantial cell lysis occurred if cells were not well chilled at this step. Cells were returned to an ice bucket for 10 min and then transferred to a petri plate containing 10 ml of HL5 medium (with penicillin at 100 U/ml and streptomycin at 100 μg/ml) and allowed to recover for 18 to 24 h. At this point cells were plated selectively as described above for calcium phosphate-mediated transformations.

Southern blot analysis. Total genomic DNA was isolated as follows. From 1×10^8 to 5×10^8 cells were washed once in 10 mM Tris (pH 8)–1 mM EDTA (TE) and then suspended in 10 ml of TE. Sarkosyl (150 μl) was added, and the sample was then extracted with an equal volume of phenol, followed by extraction with chloroform. Next, 50 μl of a 10-mg/ml stock of RNase A was added, and the sample was incubated at 37°C for 1 h, followed by addition of 50 μl of a 5-mg/ml stock of proteinase K and another 1-h 37°C incubation. Samples were then reextracted with phenol and chloroform, precipitated with ethanol, and suspended in TE for storage.

Gels (0.65% agarose) were run in TBE buffer (6). These were blotted to GeneScreen Plus and hybridized following the manufacturer's directions (Du Pont). ^{32}P-labeled probes were made by using a random-primer labeling kit (Boehringer-Mannheim).

RESULTS AND DISCUSSION

We have constructed two *Dictyostelium* hygromycin resistance (Hyg^r) vectors (Fig. 1): pDE104, which integrates into the genome of the transformed cells, and pDE109, which replicates extrachromosomally by using the *Dictyostelium* sequence Ddp2 to confer replication (9; Leiting and Noegel, in press). Both of these vectors have the actin 15 promoter fused to the coding region of the hygromycin phosphotransferase gene (*hph*) from *Escherichia coli*, with 3'-flanking sequences from the actin 15 gene situated downstream to provide termination signals. Both vectors also contain several unique restriction sites for introduction of other DNA fragments. These vectors both rely on translational fusions, with the eighth codon of the actin 15 gene fused to the third codon of the *hph* gene. The integrating vector pDE104 confers resistance to both G418 and hygromycin and was therefore used in the initial establishment of transformation conditions.

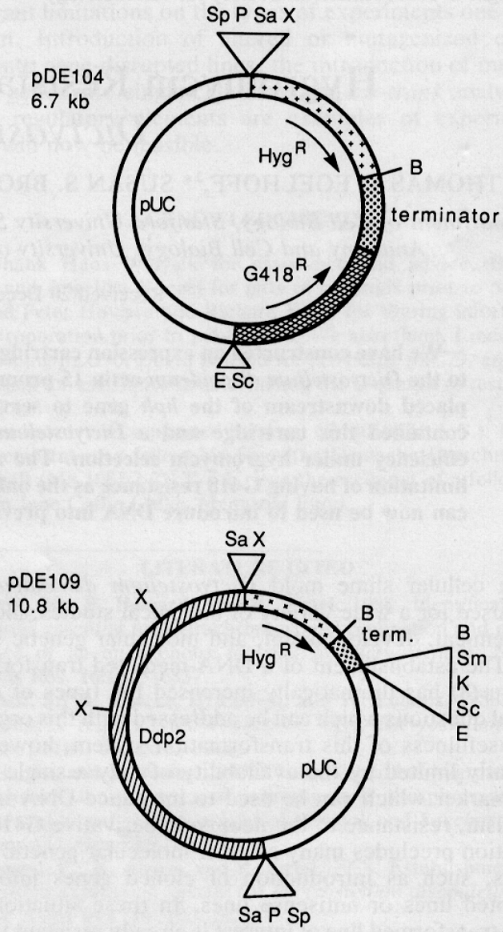

FIG. 1. Restriction maps of pDE104 and pDE109 vectors. Restriction site abbreviations: Sp, *Sph*I; P, *Pst*I; Sa, *Sal*I; X, *Xba*I; B, *Bam*HI; Sc, *Sac*I; E, *Eco*RI; Sm, *Sma*I; K, *Kpn*I. In pDE109, *Sph*I, *Sma*I, *Kpn*I, and *Sac*I are unique sites.

We introduced pDE104 into the axenic cell line Ax4 (5) by the calcium phosphate coprecipitation method (7). Colonies were obtained with both G418 selection and hygromycin selection. Under hygromycin selection, colonies became visible at 6 to 8 days, similar to the time of appearance of G418-resistant colonies with the same vector. To confirm that colonies obtained in medium containing hygromycin represented true transformants, two tests were used. First, Southern blot analysis was performed to confirm the introduction of pDE104 into the Ax4 recipient line. Southern blot analysis of four isolates confirmed the presence of the plasmid in all transformants (data not shown). Plasmid copy number between these isolates was variable, ranging from a few to about 200, as estimated by Southern blot analysis.

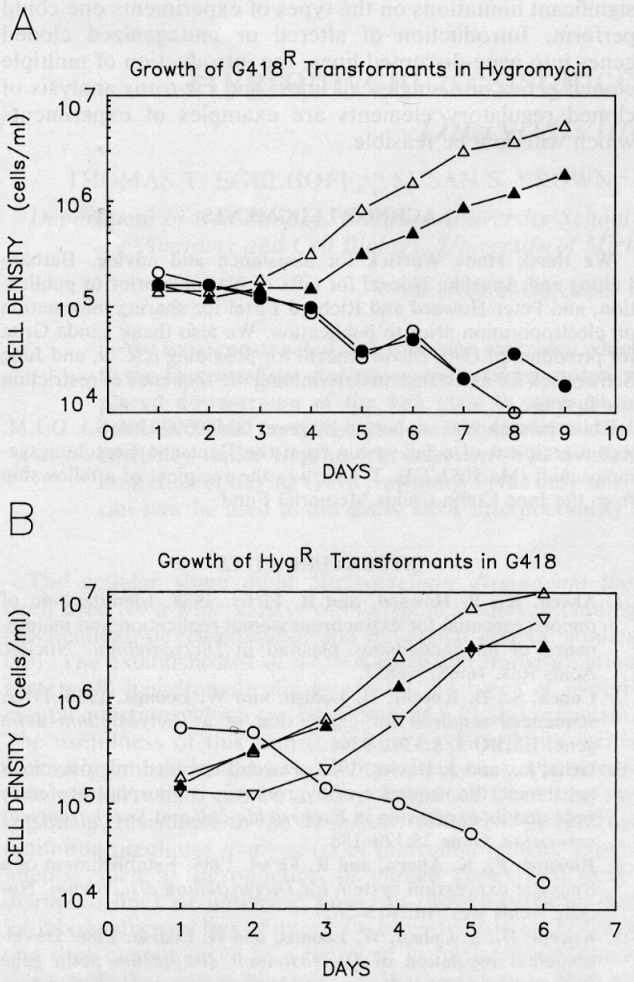

FIG. 2. Growth rates of pDE104 transformants of Ax4 cells in HL5 medium (7) containing hygromycin or G418. All tests started from log-phase cultures, inoculated on day 0 at 5×10^4 cells per ml (except the Ax4 sample in B, which started at 5×10^5 cells per ml). (A) Open and solid triangles represent two independent pDE104 transformants, and open and solid circles represent two independent pNEO-MLS (a vector containing the G418^r gene but not the Hyg^r gene) control transformants; all lines were initially isolated by G418 selection. Hygromycin was used at 50 μg/ml during the growth rate test. (B) Open triangles, solid triangles, and inverted open triangles represent three independent pDE104 transformants obtained by hygromycin selection, and circles represent the control Ax4 line. G418 was used at 10 μg/ml for the growth rate test.

The second test involved measuring the growth rates of the transformants to test for function of the linked but unselected antibiotic resistance gene. Transformants selected in G418 were tested for growth in hygromycin and vice versa. Figure 2A shows that two independent pDE104 transformants which were selected in G418 were able to grow in hygromycin. Control cell lines transformed with pNEO-MLS, a vector containing the G418^r gene but not the Hyg^r gene, failed to grow in the presence of hygromycin. In the reciprocal test, three pDE104 transformants isolated via hygromycin selection were able to grow in the presence of G418 (Fig. 2B). Untransformed Ax4 cells were unable to grow under these conditions. A comparison of Fig. 2A and B indicates that under these conditions, cells selected in hygromycin and transferred to G418 grew more rapidly (approximately 17-h doubling time) than cells selected in G418 and then transferred to hygromycin (approximately 30-h doubling time). One possible explanation for these results is that the G418 resistance cartridge functions better than the hygromycin resistance cartridge at conferring growth rates that approach that of the wild type (approximately 11 h). Furthermore, the drug concentration used for hygromycin selection may be more critical than for G418 selections.

In subsequent experiments, we were unable to obtain reproducible transformation with pDE104 via hygromycin selection. When pDE104 transformants were selected via G418 resistance, however, subsequent resistance to hygromycin was still seen. It is not clear why direct selection on hygromycin has not worked reproducibly with this plasmid. To circumvent this problem, we constructed a high-copy-number extrachromosomal plasmid containing the hygromycin resistance cartridge. This vector, pDE109, was found to transform *Dictyostelium* cells very efficiently by either electroporation or calcium phosphate-mediated transformation. Colonies became visible 6 to 14 days after hygromycin was added. Most of our characterization has been done with the axenic cell line Ax4 and electroporation; generally, 400 to 1,200 colonies were obtained when 5 μg of plasmid was used to transform 10^7 cells. pDE109 and derivatives of it have been introduced into the axenic cell lines Ax2 (10) and Ax4 (5) and into myosin-null *Dictyostelium* strains (D. Manstein, M. Titus, A. De Lozanne, and J. Spudich, EMBO J., in press). The range of hygromycin concentrations which allowed successful transformation was narrow. We found that selection with 25 to 35 μg/ml was optimum. We also found variation between experiments in resistance of the transformed cells. In many experiments, selection at 35 μg/ml gave colonies within 10 to 15 days, while in other experiments it was necessary to lower the hygromycin concentration to 25 μg/ml to obtain colonies within 10 to 15 days. It is recommended that several hygromycin concentrations be employed when establishing conditions.

Southern blots of Ax4 lines transformed with pDE109 indicate that the plasmid was present at high copy number and was extrachromosomal in the transformed lines (Fig. 3). Unrestricted and *Bam*HI-restricted pDE109 DNA purified from *E. coli* served as size controls for supercoiled monomer (lane 1, SUP) and linear plasmid mobilities (lane 2, LIN), respectively. Hybridization to unrestricted DNA from an Ax4-derived line transformed with a derivative of the integrating vector pNEO-MLS revealed the position of the uncut chromosomal DNA (lane 3, CHR). Hybridization to unrestricted DNA samples from a pDE109-transformed line indicated that the major plasmid form present was a supercoiled extrachromosomal monomer (lane 4). Higher-molecular-weight forms probably represent concatamers (Leiting and Noegel, in press). In *Bam*HI restriction digests of transformants, hybridization to an 11-kb band confirmed the presence of the intact linear plasmid (lane 5).

Colonies that were spontaneously resistant to hygromycin were sometimes seen in control transformations to which no DNA was added. Most of our characterization of this phenomenon has been done with the Ax4 cell line. In this analysis, these colonies were sometimes transient and always of low frequency (≤5% of the frequency of true transformants seen with pDE109). Therefore, we recommend that control transformations (omitting DNA) always be performed to monitor the frequency of false-positives and that dilution into microtiter plates be used to ensure clonal colonies. The cells in these colonies must then be confirmed to contain the transforming plasmid by Southern blotting.

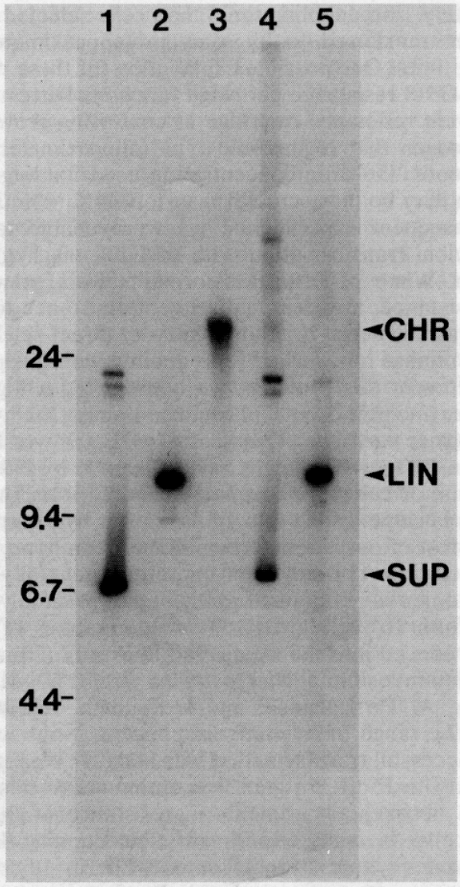

FIG. 3. Southern blot of a pDE109 transformant of Ax4 cells probed with pUC19 DNA. Lane 1 contains 5 ng of supercoiled pDE109 isolated from *E. coli*. Lane 2 contains 5 ng of pDE109 restricted with *Bam*HI. Lane 3 contains unrestricted total DNA from an Ax4 line transformed with a derivative of the integrating vector pNEO-MLS. The mobility of this sample is indicated in the right margin by CHR. Lane 4 contains unrestricted DNA from an Ax4 line transformed with pDE109. The mobility of the supercoiled monomer is indicated in the right margin by SUP. Lane 5 contains *Bam*HI-restricted DNA from the same pDE109-transformed line. The mobility of the linear pDE109 is indicated in the right margin by LIN. The mobility of linear bacteriophage lambda size standards is indicated in the left margin (in kilodaltons).

With this caveat, the vector pDE109 can be used for efficient introduction of DNA into *D. discoideum*.

The establishment of conditions by which DNA can be introduced into previously transformed cell lines opens the way for many new types of experiments. Previously, the availability of only one selectable marker ($G418^r$) placed significant limitations on the types of experiments one could perform. Introduction of altered or mutagenized cloned genes into gene-disrupted lines, the introduction of multiple cloned genes into single cell lines, and *cis-trans* analysis of cloned regulatory elements are examples of experiments which will now be feasible.

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