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Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene Under Control of the SV40 Early Region Promoter

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Summary: A bacterial gene (neo) conferring resistance to neomycin-kanamycin antibiotics has been inserted into SV40 hybrid plasmid vectors and introduced into cultured mammalian cells by DNA transfection. Whereas normal cells are killed by the antibiotic G418, those that acquire and express neo continue to grow in the presence of G418. In the course of the selection, neo DNA becomes associated with high molecular weight cellular DNA and is retained even when cells are grown in the absence of G418 for extended periods. Since neo provides a marker for dominant selections, cell transformation to G418 resistance is an efficient means for cotransformation of nonselected genes.
Key Words: Antibiotic resistance—Cell transformation—DNA transfection—Recombinant DNA—Bacterial genes.

There are two principal approaches available for the introduction of exogenous DNA into mammalian cells. Simian virus 40 (SV40) can be used as a transducing vector because it can replicate vegetatively in primate cells (1) or become integrated into host chromosomal DNA in a wide variety of cells (2). The experimental strategy has been to replace various regions of the viral genome with cloned segments of DNA and to propagate the recombinants with the aid of helper viruses in cultured animal cells (3–10). Alternatively, exogenous DNA may be introduced directly into recipient cells by either the calcium phosphate precipitation technique (11), DEAE-dextran (12), or microinjection (13,14). The pioneering experiments for this approach relied on the transformation of TK^- mammalian cell lines to a TK^+ phenotype with the herpes simplex thymidine kinase gene (15–17). Subsequently, unrelated DNA sequences have been integrated with the thymidine kinase gene in either linked or cotransformation experiments (18,19). Transformation of appropriate mutant cell lines has also been demonstrated with cellular DNA sequences and this approach has allowed the isolation of the chicken thymidine kinase gene (20) and the hamster adenine phosphoribosyl transferase gene (21). Morphologic transformation of normal cells forms the basis of current attempts to isolate cellular oncogenes (22,23).

Unfortunately, experiments that rely upon complementation of cell mutations by trans-

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duced genes are limited by the availability of mutant mammalian cell types to serve as gene recipients. Dominant-acting genetic markers, for example, those that produce a selectable change in the phenotype of normal cells, offer a solution to this difficulty. The isolation of methotrexate-resistant transformants after transfection of normal cells with DNA from drug-resistant cells (24) exemplifies this approach. However, transformation for methotrexate-resistant dihydrofolate reductase is infrequent and, therefore, the utility for cotransformation with other genes appears limited.

Recently, our laboratory devised a family of SV40 hybrid plasmid vectors to facilitate studies of gene transfer and gene expression in mammalian cells (8,25). Plasmids containing DNA segments coding for rabbit β-globin (25), mouse dihydrofolate reductase (10), and *E. coli* xanthine-guanine phosphoribosyl transferase (8) can induce the synthesis of the corresponding gene products in mammalian cells. The expression of the bacterial gene, gpt, permits the utilization of xanthine as a substrate for the purine salvage pathway and a selection can be established in which gpt functions as a dominant marker for cell transformation (26). In this paper, we describe a second bacterial gene which, when incorporated into the same family of plasmid vectors, also provides a dominant selective marker for transformation of cultured mammalian cells.

The selection for transformation of mammalian cells relies on cell killing by an aminoglycoside antibiotic, G418 (27). The structure of G418 resembles gentamicin, neomycin, and kanamycin (28) but, unlike these related compounds, G418 interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells (27). These aminoglycoside antibiotics can be inactivated by the bacterial phosphotransferases, APH(3′)II and APH(3′)I encoded by transposons Tn5 and Tn601, respectively (29). Jiminez and Davies (30) showed that yeast could be genetically transformed to G418 resistance by the phosphotransferase gene contained in Tn601 DNA. Thus, it seemed feasible (suggested by Sydney Brenner) that the acquisition and expression of the phosphotransferase gene by mammalian cells might confer resistance to G418 toxicity. Accordingly, the phosphotransferase gene from Tn5 (designated here neo) (29,31) was introduced into the mammalian transcription unit of the pSV plasmid vectors. In this arrangement the SV40 early promoter is 5′-proximal, and an intron and polyadenylation signal are 3′-proximal, to the neo gene (8,25). Transfection of a wide variety of mammalian cell lines with these pSV-neo recombinants yields stable transformants that are resistant to G418 at a frequency of one transformant per 10^4–10^5 transfected cells. Our data suggest that transformation results from the acquisition, maintenance, and continued expression of the neo gene in the cellular genome. Concurrent with our experiments, Colbère-Garapin et al. (32) achieved mammalian cell transformation to G418 resistance with recombinant DNA containing the Tn5 neo gene linked to the herpes thymidine kinase promoter DNA segment.

MATERIALS AND METHODS

Cells

A wide range of cultured mammalian cells are sensitive to the antibiotic G418 (Table 1) and several of these have been used for transformation experiments. The cells were routinely maintained in Dulbecco-modified Eagle medium containing 10% newborn calf serum, penicillin and streptomycin, and the indicated concentrations of G418 were added to the medium.

Enzymes

Restriction enzymes were purchased from New England Biolabs and Bethesda Research Laboratories and digestions were performed according to the supplier's specifications. T4 polynucleotide kinase was purchased from New England Nuclear and S1 nuclease from Boehringer Mannheim. T4 DNA ligase and *E. coli* DNA polymerase I were kindly provided by S. Scherer, Stanford University.

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Antibiotic G418

Samples of antibiotic G418 were generously provided by Dr. P. J. L. Daniels of Schering Corporation. Stock solutions containing 4 mg/ml G418 in 100 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulphonic acid buffer, pH 7.3, were stored at −20°C and added in appropriate amounts to the cell culture medium. The G418 concentration refers to the actual amount of drug in the solution and takes into account that the solid material was only 40–50% G418.

DNA Transfection and Selection of Transformed Cells

Supercoiled plasmid DNA, without added carrier DNA, was introduced into tissue culture cells (10 μg for approximately 5×10^6 cells) using the calcium phosphate precipitation technique (11) with the addition of a glycerol shock after 4 h (33). About 48 h after exposure to DNA, the cells were trypsinized and replated at a 1:20 dilution. Within 12–16 h, G418 was added to the medium at a concentration of 400 μg/ml. The medium plus drug was changed every 4 to 5 days. Colonies were first detected after about 7 days in the selective medium and, 7–13 days later, independent colonies were trypsinized in cloning cylinders and transferred to microtiter wells. When the colonies were small, the transplanted cells were grown nonselectively for an initial 2–3 days. Once established, the clones were expanded to stable cell lines in medium containing 400 μg/ml G418. In some instances, the initial selection and subcloning used 400 μg/ml of G418 but the transformed cells were subsequently maintained in 200 μg/ml of G418.

The selection strategy of permitting cell growth prior to the addition of G418 was adopted because a significant reduction in the transformation frequency occurred if G418 was added before 48 h. The transfected cells were replated at lower cell density because G418 is most effective against dividing cells. Consequently, if cells become stably transformed early after transfection, cell division prior to selection may result in overestimation of the transformation frequency. Nevertheless, none of the transformants from randomly selected colonies appeared to have the same organization of the integrated pSV-neo DNA.

Analysis of Transformed Cell DNAs for pSV-neo Sequences

High molecular weight cellular DNA was extracted as described by Wigler et al. (17), incubated with an excess of restriction enzyme, and the digests were separated by electrophoresis in 0.8% agarose gels. After a mild depurination reaction (34), the DNA was transferred from the gel to diazobenzyloxymethyl paper (DBM paper) (35), hybridized with radioactively labeled DNA probes (36), and radioautographed using Kodak XR5 film and Cronex lightning fast intensification screens at −70°C (37).

Analysis of Cytoplasmic RNA Extracted from pSV2-neo Transformed Cells

Cytoplasmic RNA was extracted from semiconfluent cultures of transformed cells as described previously (38). The RNA was separated from contaminating DNA by pelleting through cesium chloride and then the poly A^+ RNA fractions were characterized using the Weaver-Weissmann variation (39) of the Berk-Sharp procedure (40). DNA hybrization probes (shown with individual experiments) were prepared by labeling appropriate restriction fragments at their 5′-ends with [γ-^{32}P]-adenosine triphosphate and polynucleotide kinase (41). The DNA probes were hybridized with RNA samples under conditions of DNA excess, RNA-DNA hybrids were digested with S1 nuclease, and the protected fragments were analyzed by gel electrophoresis (39,40).

Protein Labeling and Immunoprecipitation Reactions

Semiconfluent plates of pSV2-neo transformed cell lines were labeled for 14 h at 37°C

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with [^{3}H]leucine (200 μCi/plate, specific activity 55 Ci/mmol, New England Nuclear Laboratory). The soluble proteins were extracted from approximately 2×10^7 cells (7) and immunoprecipitated with an antiphosphotransferase APH(3′)II serum (provided by J. Davies, Geneva). *Escherichia coli* cells (HB101) containing plasmids were grown to approximately 2×10^8 cells/ml in M9 minimal medium plus glucose with supplements of leucine, proline, threonine, and thiamine. Samples of the cultures (0.5 ml) were washed and resuspended in the same medium lacking leucine and then [^{3}H]leucine was added (200 μCi/ml) for 60 min at 37°C. Excess unlabeled leucine was added and, after washing in M9 medium, the cells were disrupted by sonication. Cell debris was removed by centrifugation at 14,000 *g* for 10 min and the supernatant was used directly for immunoprecipitation reactions. After incubation overnight at 0°C, the immune complexes were adsorbed to inactivated *S. aureus* cells (IgGsorb, Enzyme Center, Boston) and removed by centrifugation (42). The *S. aureus* cells were washed extensively and the bound proteins were eluted and electrophoresed in SDS polyacrylamide gels (43). After electrophoresis, the gels were treated with EN^{3}HANCE (New England Nuclear Laboratory), dried and autoradiographed as described above.

RESULTS

Mammalian Cells Are Sensitive to G418

The sensitivity of various cultured cell lines to G418 was assessed by plating cells at low cell density in microtiter wells in a medium supplemented with various concentrations of G418. Even at the highest drug concentration tested (800 μg/ml), sensitive cells divided once or twice before cytotoxicity was observed. The response time for cell killing appears to correlate with growth rate, since the most rapidly growing cells are killed in the shortest intervals. At lower concentrations of G418 (100 μg/ml) there is a significant delay but the cells are killed eventually. All of the cell lines that have been tested (Table 1) are killed by G418 but CV1 and HeLa are unusual because, at high cell density, these cells may require 10–14 days in G418 (400 μg/ml) before the cell killing can be observed.

TABLE 1. *G418-sensitive mammalian cell lines*[a]

Mouse	Monkey	Human
L	CV1	LNSV
Ltk$^-$	CV1-P	HeLa
3T3	TC7	K-562
3T6	COS	
PCC4		
F9		
MEL		

[a] So far, no cell line has been found that is naturally resistant to G418.

Construction of Recombinant Plasmids Containing neo

The bacterial transposon Tn5 encodes a gene (neo) whose protein product—a phosphotransferase (APH(3′)II)—confers resistance to the kanamycin-neomycin group of antibiotics (31). From the studies of the organization of Tn5 DNA, Reznikoff and colleagues (44,45) were able to identify the DNA segment that is essential for the expression of neo. The Col E1:Tn5 plasmid pRZ112 (44), a deleted form of the original Col E1:Tn5 hybrid plasmid (pRZ102, Fig. 1), was the source of the neo DNA segment. pRZ112 DNA was digested to completion with *Hin*cII endonuclease and the 2.5 kb neo DNA segment was obtained by agarose gel electrophoresis. After ligating a decanucleotide sequence containing the *Bam*HI restriction site (Collaborative Research) to the ends of the neo segment (46) the mixture was digested with an excess of *Bam*HI and *Hin*dIII restriction endonucleases and the resulting 1.4 kb neo DNA fragment was purified by gel electrophoresis. This fragment, containing *Hin*dIII and *Bam*HI cohesive ends at the 5′- and 3′-ends, respectively, was inserted between the *Hin*dIII and *Bam*HI restriction sites in pBR322 DNA. The resulting plasmid, pBR-neo (Fig. 1), confers resistance in *E. coli* to both ampicillin and

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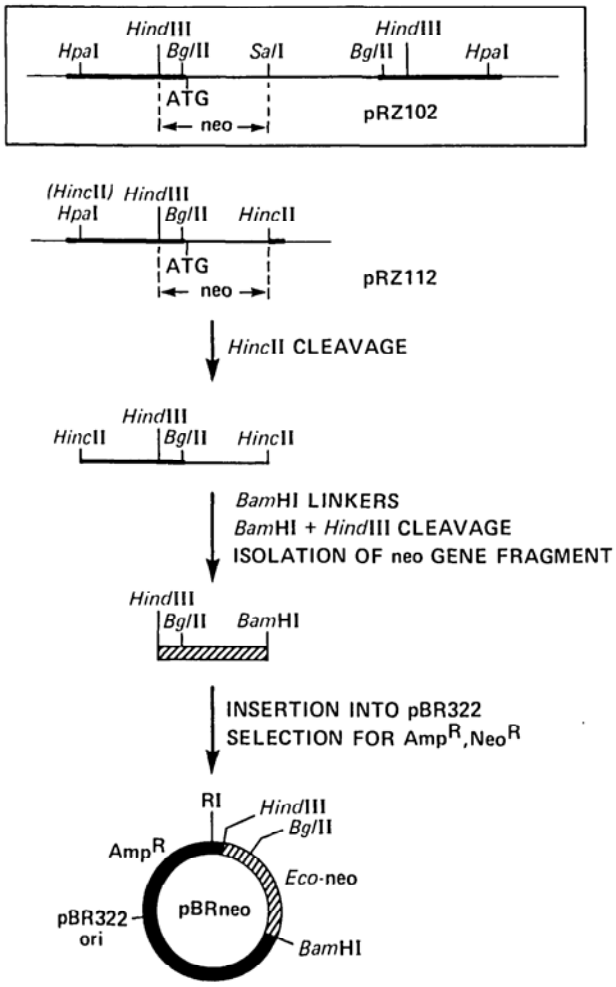

FIG. 1. Organization of transposon Tn5 and scheme for the construction of recombinant plasmid pBR-neo. The plasmid pRZ102 contains a complete copy of Tn5 inserted into ColE1. The inverted repeat sequences at the ends of the transposon are shown as thick lines. The region essential for expression of neomycin resistance in *E. coli* (neo) is indicated together with the ATG codon that initiates the coding sequence of the phosphotransferase. Relevant restriction endonuclease recognition sites are included in the diagram. pRZ112 was derived from pRZ102 by partial digestion with *Hin*cII endonuclease and ligation to eliminate a large segment of Tn5 DNA to the 3′ side of neo (*Hin*cII endonuclease cleaves at *Sal*I and *Hpa*I endonuclease recognition sites.) Details of the manipulations involved with the construction of pBR-neo are given in the text.

neomycin; since the neo DNA segment interrupts the tetracycline resistance gene, cells carrying this plasmid are sensitive to this antibiotic.

The cloned neo DNA segment was readily introduced into the plasmid vector—pSV2 (8) (Fig. 2) by excising the β-globin cDNA segment from pSV2-βG (25) with *Hin*dIII and *Bgl*II endonuclease digestion and substituting the neo fragment via the corresponding cohesive ends. The pSV3-neo and pSV5-neo (Fig. 2) derivatives were constructed from pSV2-neo as previously described (8,25). Each of the pSV-neo plasmids replicates efficiently in *E. coli* strain HB101 and confers resistance to ampicillin and neomycin. Cloned isolates of each of the recombinant plasmids were shown to have the anticipated structures by appropriate restriction enzyme analyses (data not shown).

Cell Transformation with Recombinant Plasmids Containing neo

Transfections of Ltk$^-$ cells with pRZ112 or pBR-neo plasmid DNA yielded occasional G418-resistant colonies (a frequency of about one transformant in 5×10^6 transfected cells). However, with 3T6 cells as recipient no transformants have been recovered with pRZ112 DNA and only one transformant has been isolated after transfection with pBR-neo (frequency about one transformant in 10^7 transfected cells). In control experiments, cells transfected with either pSV2-gpt (8), pSV2-βG (25) DNA, or mock-transfected without DNA have never yielded G418-resistant colonies (frequency less than 1 transformant in 10^7 transfected cells).

In contrast to the low frequencies of G418-resistant transformation with pRZ112 (ColE1-neo) or pBR-neo plasmid DNAs, several different mammalian cell lines were transformed to G418 resistance at relatively high frequency with the pSV-neo plasmid derivatives (about one transformant in 10^4 to 10^5 transfected cells). Although the transformation frequencies with the different pSV-neo recombinants fall within a relatively narrow range, there is a consistent small difference which reflects the plasmid's potential for replication in different host cells (Table 2). For example, the frequency of stable G418-resistant monkey cell transformants is two- to three-fold lower with pSV3-neo than with pSV5-neo, whereas the converse applies to the formation of G418-resistant mouse cell transformants. This differ-

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