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DNA-mediated gene transfer into epidermal cells using electroporation

M. Reiss[1], M.M. Jastreboff[2], J.R. Bertino[1,2], and R. Narayanan[2]

Departments of Medicine[1] and Pharmacology[2], Yale University School of Medicine, New Haven, CT 06510

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Summary: A reliable method for the introduction of foreign DNA into epidermal cells is described. Electroporation of murine BALB/c MK-1 epidermal cells with pSV2-CAT resulted in the transient expression of chloramphenicol-acetyltransferase (0.03 to 0.05 nmoles acetylchloramphenicol per mg protein per min) in the transfected cells. Transfection of MK-1 cells with pSV2-neo led to the appearance of approximately eight G418 resistant clones per 10^{-6} cells per µg of plasmid DNA. Distinct patterns of integration of SV2-neo were detected in three different resistant clones.

We have recently embarked on a systematic study of the phenotypic alterations in terms of growth requirements and characteristics of differentiation of epidermal cells by the introduction of selected retroviral oncogenes. A prerequisite for a study of this type is the availability of an efficient method for gene transfer into epidermal cells. In this paper, we show that successful DNA-mediated gene transfer leading to transient as well as stable gene expression in murine keratinocytes can be achieved using electroporation (1). Although this technique has been applied successfully for gene transfer into hematopoietic and mesenchymal cells, as well as into rat hepatocytes (1,2,3), transfection of cloned DNA molecules into epidermal cells has not been previously reported.

METHODS

Cell culture

BALB/c MK-1 cells (4) were propagated in plastic tissue culture flasks, in medium consisting of three parts of calcium-free Dubecco's modified Eagle's medium and one part Ham's F12 medium (both from GIBCO). This mixture was supplemented with 5% (v/v) fetal bovine serum (GIBCO), epidermal growth factor (10 ng/ml), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml) (all obtained from Collaborative Research, Lexington, MA.), choleratoxin (1 mM) (List Biological Co., Inc., Campbell, CA), and thyroxine (2 nM) (Sigma), and contained gentamicin (50 µg/ml) (GIBCO).

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Transfection of plasmid-DNA

Keratinocytes were transfected with pSV2-CAT (obtained from Bruce Howard, NCI) linearized with Pvu 1, or pSV2-neo (BRL, Bethesda, MD), linearized with Eco R1, in order to increase the transfection efficiency (1). Cells from preconfluent cultures were detached by trypsinization and resuspended in 0.5 ml of ice-cold Ca^{2+} and Mg^{2+} free phosphate-buffered saline, to a final concentration of 1 to 5 x 10^7 cells/ml. Five to 50 µg of cesium chloride purified plasmid DNA was added to 0.5 ml of cell suspension and incubated for 10 min at 0°C. The mixture was transferred to sterile polysterene cuvettes provided with two aluminium electrodes as described by Potter et al. (1). An electric pulse of 2.0 kV was then applied to the electroporation chamber by an ISCO model 494 electrophoresis dc power supply, with the current and wattage dials set at 5% of the maximum values (1). The cell suspension was kept at 0°C for 10 min, diluted with prewarmed fresh medium, and transferred to a new flask after incubation for 10 min at room temperature.

Assay of CAT activity

Chloramphenicol 3-O-acetyltransferase (EC 2.3.1.28, CAT) activity was determined in cell sonicates prepared at 72 h after electroporation as described by Gorman et al. (5). The assay mixture (final volume: 0.1 ml) contained 4 mM acetyl coenzyme A, 1 µCi of [^{14}C]-labeled chloramphenicol (40-60 mCi/mmol; New Engl. Nuclear), and 0.08 mg/ml of protein in cell lysates, in 0.01 M Tris-HCl (pH 7.5). Following incubation at 37°C for 1 h, the acetylated reaction products were separated by thin layer chromatography. After identification of reaction products by autoradiography at -70°C, spots were scraped off the plates, and radioactivity determined by scintillation spectrometry (5). Protein concentration in the cell extracts was determined as described by Bradford (6).

Determination of stable integration of the neo^R gene in keratinocyte clones

At 24 h after electroporation, the culture medium was entirely replaced by fresh medium. Twenty four to 48 h later, cells were detached by trypsinization, counted, and plated at a density of 0.8 to 3.5 x 10^4 cells/cm^2 in fresh medium containing 150 µg/ml Geniticin (G418, GIBCO). After selection in G418 for 14 to 21 days, isolated colonies were picked using cloning cylinders, expanded in mass culture, and genomic DNA isolated as described (7). Aliquots of 20 µg of DNA were digested to completion with EcoR1, or Bam H1 plus Hind III (New England Biolabs, Beverly, MA.), subjected to electrophoresis on 1.0% (w/v) agarose gels, and transferred to nitrocellulose filters as described by Southern (8). Filters were hybridized with nick-translated pBR322 or a 1.4 kb Bam H1-Hind III neo-specific DNA fragment as probe (Pneo, PL Biochemicals). Filters were washed at 68°C in 0.1X standard saline citrate solution (9), and hybridizing sequences detected by autoradiography at -70°C (9).

RESULTS

Determination of transient expression of SV2-CAT was performed as a rapid assay for transfectability of MK-1 cells using electroporation. CAT activity in cell extracts obtained 72 h after transfection of keratinocytes with pSV2-CAT is shown in Figure 1. Thin layer chromatography revealed a diacetylated (a) as well as two monoacetylated (b and c) metabolites of [^{14}C]-chloramphenicol in the transfected MK-1 cells (Fig. 1, lane 1). Approximately 2 to 3% of the [^{14}C]-

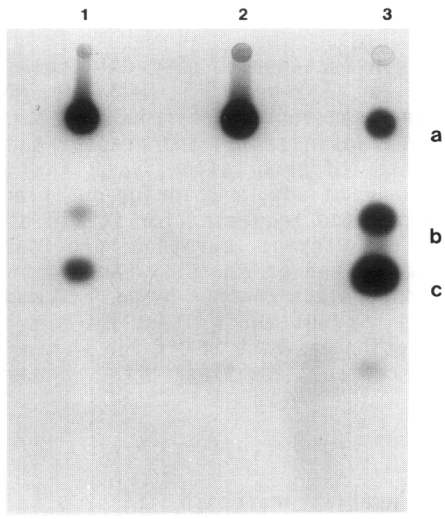

Figure 1. Autoradiography of thin layer chromatogram of CAT assay. Lane 1: CAT activity in MK-1 cells 72 h after electroporation with pSV2-CAT DNA. Lane 2: CAT activity in mock transfected MK-1 cells. Lane 3: [^{14}C]-chloramphenicol incubated with CAT enzyme.

chloramphenicol was converted to acetylated metabolites, corresponding to an enzyme activity of 0.03 to 0.05 nmoles acetylchloramphenicol per mg protein per min. No CAT activity was detected in mock transfected cells (Fig. 1, lane 2).

In order to determine whether electroporation could be applied to achieve stable integration of foreign DNA into murine epidermal cells, we used the dominant selectable marker gene SV2-neo (10), which confers resistance to the antibiotic geneticin (G418), a neomycin analogue toxic to eukaryotic cells. Preliminary experiments showed that G418 was lethal to 90 to 100% of sensitive keratinocytes at a concentration of 150 µg/ml. Varying amounts of EcoR1 linearized pSV2-neo DNA were transfected into MK-1 cells by electroporation, and the number of drug resistant colonies counted 21 days later (Table 1). The efficiency of transfection was linear up to 10.4 µg plasmid DNA/10^7 cells (Table 1). The plating efficiency of MK-1 cells is approximately 1% (4). When 1 µg of

Table 1. Transfection of pSV2-neo into BALB/c MK-1 cells

Plasmid DNA (µg/10^7 cells)	Transfection efficiency (G418 resistant clones/10^6 cells plated)
0	0
1.35	7.26
10.4	80.0

plasmid DNA was used to transfect 10^6 MK-1 cells, approximately 8 clones of G418 resistant cells arose in a population of 1000 clonogenic cells exposed to the drug. Figure 2 shows a Southern blot of 20 µg aliquots of genomic DNA extracted from 3 different G418 resistant clones as well as non-transfected cells, digested with either EcoR1, or in a double digestion of Bam H1 with Hind III, and hybridized to either pBR322 (Fig. 2A), or Pneo (Fig. 2B). Both probes detected the same distinct pattern of bands in Eco R1 digests of DNA of each of the transfectants, indicating that SV2-neo sequences were integrated at multiple different sites in the genome of each clone. Hybridization of BamH1-Hind III restricted genomic DNA with Pneo revealed a single band of 2.4 kb in each of the three clones (Fig. 2B), as predicted from the structure of pSV2-neo (10). Clone #12 is exceptional in the sense that multiple copies of pSV2-neo were detected in its DNA, as evidenced by the increased intensity of the 2.4 kb band detected by Pneo in the Bam H1 and Hind III digest (Fig. 2B, lane 3), whereas non-specific hybridization of pBR322 with a band of 4.3 Kb was equally intense in all four samples (Fig. 2A). The light-microscopic appearance of colonies of transfected MK-1 cells was indistinguishable from that of those not subjected to electroporation (data not shown).

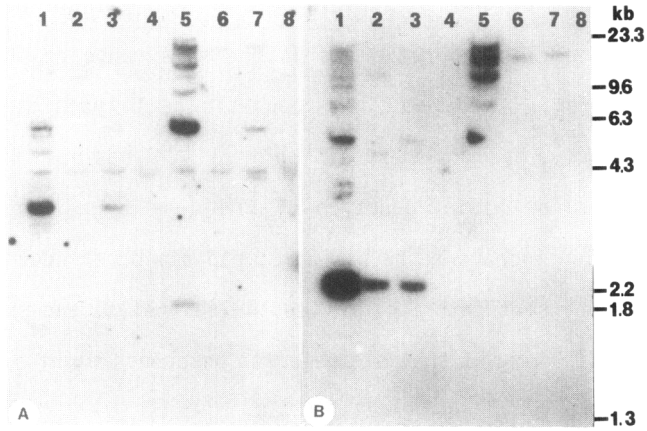

Figure 2. Southern blot of three separate G418 resistant cell clones and control cells, hybridized with pBR322 (A), and Pneo (B). Genomic DNA (20 µg per lane) of clones #12 (lanes 1 and 5), #7 (lanes 2 and 6), #3 (lanes 3 and 7) and of mock transfected cells (lanes 4 and 8) was restricted with Bam H1 and Hind III (lanes 1,2,3,4 in Fig. 2A, and lanes 1,2,3,4, in Fig. 2B), or with EcoR1 (lanes 5,6,7,8, in Fig. 2A, and lanes 5,6,7,8 in Fig. 2B). Lambda DNA digested with Hind III served as molecular weight markers.

DISCUSSION

This study demonstrates that cloned genes can be introduced into and expressed in murine epidermal cells. Stable integration and expression of at least one exogeneous gene in murine keratinocytes was achieved, and we are currently investigating the transfectability of normal human keratinocytes. Primary cultures of murine epidermal cells are used extensively for the study of chemical carcinogenesis (11). Phenotypic changes suggesting a preneoplastic state have been induced by infection of such cells with mouse sarcoma viruses (4,12,13), and similar results have recently been obtained using human keratinocytes as targets (14). However, these studies do not address the possible contribution of retroviral sequences to the changes observed. Therefore, the availability of a reliable method to introduce cloned foreign DNA into epithelial cells is necessary in order to be able to determine the pathogenetic effects of retroviral oncogenes. Yoakum et al. (15) have reported on the transfectability of v-Ha ras and SV2-neo DNA into human bronchial epithelial cells using protoplastic fusion. Transfection of the neo^R gene yielded one G418 resistant clone per 1000 cells (15). Our results compare favorably with those using protoplastic fusion, as we detected up to 8 drug-resistant clones per 1000 clonogenic cells per microgram of plasmid DNA. Thus, the transfection frequency we obtained by using electroporation is in the same range as that reported by Potter et al. (1) for a variety of hematopoietic cell types as well as mouse fibroblasts. Recently, transient expression of SV2-CAT has been found in primary rat hepatocytes, following introduction of the gene by electroporation (3). Thus, electroporation appears to be a simple, rapid and reproducible method for DNA mediated gene transfer into cultured epithelial cells, and should therefore allow the systematic study of the role selected oncogenes might play in inducing (pre) malignant changes in these cells.

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