High-Frequency Transfection of Mouse FM3A Mammary Carcinoma Cells in Suspension Culture with Plasmid DNA

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ABSTRACT. High-frequency transfection of mouse FM3A cells, grown in suspension, with plasmid pSV2neo DNA was achieved by incubation of the cells with DNA plus polybrene for 6 h followed by an osmotic shock with a hypertonic NaCl solution. When incubated for 20 min at 34°C, FM3A cells showed resistance to the osmolarity change from 0.1 to 9.0% NaCl in the medium. Within this concentration range, 5-7% gave the highest efficiency of transfection. Both linear and circular forms of plasmid DNA produced transformants with equal efficiency. This method was simple, reproducible, and carrier DNA was not required. The efficiency was about 100 times higher than that of the method with DNA-calcium phosphate precipitates. Transformed cells were stable and different numbers of plasmid DNA copies were detected.

Recent development of methods of DNA-mediated gene transfer, together with the molecular cloning of genes and DNA-sequencing, has made it possible to identify various genes of mammalian cells. Many genes coding for enzymes have been isolated by complementation of the defective gene in somatic cell mutants (10, 15), oncogenes by alteration in growth properties of the transfected cells (22), and a gene coding for cellular growth factor (25) and one for receptor (13) by detecting the products expressed in the transfected cells. For DNA transfection to cultured mammalian cells, the DNA-calcium phosphate method (6, 14) has been widely used. This method has been shown to be simple, reproducible, and highly efficient. However, the rate of gene-transfer by this method is highly dependent on the cell line used as the recipient.

Mouse mammary carcinoma FM3A cells have been established as a suspension culture and are frequently used for studies of somatic cell genetics. Many types of mutants have been isolated from this cell line and characterized by properties such as altered sensitivity to drugs (1, 12), nutritional requirements (2, 3), or temperature-sensitivity (2, 8, 19, 24). However, since the DNA-calcium phosphate method does not usually result in high-efficiency transfection with this cell, further analysis of interesting mutants at the molecular level has been prevented.

Other methods of gene-transfer include the use of DEAE-dextran (17), liposomes (18), and protoplast fusion (21). However, these methods are not suitable for mouse FM3A cells because of the low frequency of obtaining stable transformants. High-voltage electric pulses seemed to be successful (7), but this method requires special, expensive equipment. Recently Kawai and Nishizawa (11) and Chaney *et al.* (5)

reported a simple method of transfection by treatment of cells with DNA plus polybrene followed by a high concentration of dimethyl sulfoxide (DMSO). We have examined this method and found that the frequency of transfection was better than that of the DNA-calcium phosphate method. To improve this method, we tried several alternatives to DMSO treatment to increase the cell permeability. Our results show that osmotic shock with a hypertonic solution, 5–7% NaCl in medium, causes high-frequency transfection.

MATERIALS AND METHODS

Cells and plasmid DNA. FM3A cells, F28-7 clone, derived from mouse mammary carcinoma (12), were grown in suspension in Eagle's minimum essential medium (MEM, Nissui Seiyaku Co.) supplemented with 5% calf serum (GIBCO) and 0.1% Bactopeptone (Difco Lab.) in an atmosphere of 5% CO₂ at high humidity. Incubation of cells was at 34°C.

The plasmid pSV2neo DNA (23) has a promoter sequence of virus SV40 ligated to the bacterial aminoglycosyl 3' phosphotransferase (neo) gene which to bacteria confers resistance to neomycin/kanamycin and to mammalian cells resistance to the antibiotic G418 (Geneticin, GIBCO). Plasmid DNA was prepared from plasmid-harboring *E. coli* (HB 101) by the alkaline lysis method (16) and purified by CsCl-ethidium bromide equilibrium centrifugation (16). Plasmid DNAs were linearized by digestion with restriction endonuclease EcoR1 (Takara Shuzo Co.), and the products were confirmed by agarose gel electrophoresis (16).

DNA transfection and selection of transformants. Log-phase cells were collected by centrifugation and aliquots of 1×10^6 cells were suspended in 1 ml of culture medium in a plastic tube (Falcon tube 2095). Then 30 µg of polybrene (Aldrich, dissolved at 1 mg/ml in culture medium) and 1–10 µg of DNA (dissolved at 0.5–1.0 mg/ml in 10 mM Tris-Cl, 1 mM EDTA, pH 7.6) were added to the cell suspension. The cells were incubated at 34°C for 6 h in a CO₂ incubator with occasional gentle mixing. After incubation, cells were collected by a brief centrifugation and then suspended in 3 ml MEM or Hanks' solution which contained 5% NaCl (final concentration) but no serum. MEM and Hanks' solution gave the same results. Cells were incubated for a further 20 min at 34°C, collected by centrifugation, and suspended in 5 ml of culture medium. The cell suspension was transferred to a 60 mm plastic dish and incubated for 48–72 h in a CO₂ incubator.

For the selection of transformants, $1\text{--}3 \times 10^5$ surviving cells were plated in a 60 mm plastic dish with agar medium which contained 0.3% agar and 0.5 mg/ml G418 (as an active agent) in MEM supplemented with 10% calf serum. Agar plates were incubated for 2 weeks and colonies were scored. With this selection procedure, fetal calf serum could not be used since some small colonies developed from cells which had not been transfected with DNA. No such background was observed when calf serum was used.

Characterization of transformed cells. To test the stability of the transformed cells, colonies in the agar plates were transferred to culture medium which contained 0.5 mg/ml of G418 and incubated for 2 weeks. Then the cells were maintained in the culture medium without selection for 2 months. Sensitivity of these cells to G418 was examined by plating 1×10^4 cells in a plastic multiwell dish which contained 1.5 ml of the culture medium and variable amounts of G418. The plates were incubated for 6 days and the cell numbers were determined.

To examine the plasmid DNA sequence integrated in the chromosomal DNA, Southern

blot hybridization was performed. Cellular DNAs were extracted from isolated nuclei as described by Jariwalla *et al.* (9). DNAs were digested at 37°C for 2 h with restriction enzyme EcoRI (6 units/ μ g DNA). Digested DNAs (15 μ g/lane) were electrophoresed in 0.6% agarose gel, stained with ethidium bromide, photographed, denatured, and blotted onto nylon membrane (Hybond-N, Amersham) by electrophoresis. pSV2neo DNA (25 ng) was labeled overnight at room temperature with random primers (Amersham Multiprime DNA Labeling System) and 55 μ Ci of [α - 32 P]dCTP (3,000 Ci/mmol). Hybridization was performed overnight at 65°C in Denhardt's solution (16) containing 0.5% sodium dodecyl-sulfate (SDS) and 20 μ g/ml of heat denatured salmon sperm DNA. The membrane was washed at 65°C three times in 2 $\times$ SSC (SSC; 0.15 M NaCl, 0.015 M sodium citrate) containing 0.5% SDS, and once in 0.1 $\times$ SSC containing 0.5% SDS. Dried membrane was exposed to Kodak XAR-5 film at -70°C for about 80 h with an intensifying screen.

RESULTS AND DISCUSSION

The method of transfection with DNA-calcium phosphate precipitates, which is widely used with many cell lines, was quite inefficient with mouse FM3A cells. As shown in Table 1, it gave only 3×10^{-6} G418-resistant transformants with 5 μ g of linear pSV2neo DNA. The reason for this is not known, but the method seems to depend on the ability of the cells to take up the precipitates by phagocytosis. Since FM3A cells grow in suspension, their phagocytotic activity may be reduced. Sufficient contact of cells with the precipitates seems to occur on the surface of the plastic dish as observed microscopically. The same inefficiency of this method for FM3A cells has been reported by Hama-Inaba *et al.* (7).

We therefore examined the transfection method with polybrene as described by Kawai and Nishizuka (11) and Chaney *et al.* (5). This method employed incubation of the cells with DNA plus polybrene followed by treatment with 30% DMSO. However, FM3A cells were much more sensitive to DMSO than the cells used in those

TABLE 1. TRANSFECTION OF MOUSE FM3A CELLS WITH pSV2NEO DNA BY DIFFERENT METHODS

Exp	DNA	Treatments		G418 ^r cell per 10 ⁶
		1st	2nd	
1	linear	DNA+Ca-phosphate	10% DMSO	3
2	linear	DNA+Ca-phosphate	5% NaCl	14
3	linear	DNA+polybrene	15% DMSO	42
4	linear	DNA+polybrene	50% PEG 6000	46
5	linear	DNA+polybrene	5% NaCl	284
6	supercoil	DNA+polybrene	5% NaCl	310
7	-DNA	polybrene	5% NaCl	0
8	linear	5% NaCl, 20 min	DNA+polybrene	0
9	linear	DNA+polybrene	5% NaCl→10% DMSO	14
10	linear	DNA+polybrene	5% NaCl→50% PEG	9

In experiments 1 and 2, DNA-calcium phosphate precipitates were produced by the method of Lewis *et al.* (14). The precipitates were added to the cells and the mixture was incubated for 8 h. Then the cells were treated with 10% DMSO for 30 min (Exp. 1) or with 5% NaCl in MEM for 20 min (Exp. 2). In experiments 3-10, cells were treated as described in MATERIALS AND METHODS except that some specifications were added as described in the Table.

Fig. 1. Effect of NaCl concentration on cell growth and transfection frequency. The protocol of transfection with 5 μ g of linear pSV2neo and selection of the transformants were as described in MATERIALS AND METHODS. After incubation with DNA plus polybrene for 6 h, the cells were treated with Hanks' solution containing different concentrations of NaCl as shown in the figure. For determination of cell growth, 1×10^4 cells were incubated for 6 days and the cell number was determined. ●: Cell number; △: number of transformants per 10^6 cells.

studies, and 15–18% was practically the maximum concentration at which the cells survived. When the polybrene method with 15% DMSO was tested, the efficiency of transfection was 4.2×10^{-5} . To achieve more efficient transfection, we tested several other methods which may alter cell permeability to DNA. After incubation of cells with DNA plus polybrene for 6 h, cells were treated either with a hypertonic solution (5% NaCl in MEM), a method used to permeabilize cells to small molecules without loss of viability (4), or with a solution containing 50% polyethylene glycol (PEG), which has been used for cell fusion (20).

As shown in Table 1, treatment of cells with the hypertonic solution resulted in high-frequency transfection (2.8×10^{-4}). Treatment with PEG 6000 showed the same degree of transfection as 15% DMSO. By the standard method of DNA plus polybrene and 5% NaCl treatment (MATERIALS AND METHODS), both linear and supercoiled DNA was taken up by the cells with similar efficiency. No transformant was produced without DNA or when the order of the protocol was reversed. When cells treated by the standard method were further treated with 10% DMSO or 50% PEG, the frequency of transfection was greatly reduced. Treatment of the cells with 5% NaCl after incubation with DNA-calcium phosphate precipitates caused a several fold increase in the efficiency compared to treatment with 18% DMSO.

We next investigated the effect of various concentrations of NaCl on cell survival and transfection frequency. Figure 1 shows the results. When incubated for 20 min at 34°C, FM3A cells resisted NaCl in a range of 0.1–9.0% in MEM. Cell viability was lost abruptly in the hypotonic solution at a concentration of 0.1% (Fig. 1A) and gradually under the hypertonic conditions (Fig. 1B). When the transfection was performed with different concentrations of NaCl after treatment with DNA plus polybrene, there was a small increase in efficiency with hypotonic solution, 3×10^{-6} at 0.2% NaCl (Fig. 1A), but the highest efficiency was obtained at 0.6% NaCl ($3.2 \times$

Fig. 2. Effect of DNA amounts on transfection frequency. Different amounts of linear pSV2neo DNA were used for transfection.

10^{-4}) (Fig. 1B).

Under standard conditions, the frequency of transfection was proportional to the DNA dose, as shown in Fig. 2. These results indicate that carrier DNA was not required and transformation depended solely on the input DNA. At a dose of $10 \mu\text{g}$ of plasmid DNA, a frequency of 7.5×10^{-4} was obtained.

To test the stability of the transformed cells, independent colonies were transferred to the culture medium and first incubated with G418 for 2 weeks and then without

Fig. 3. Sensitivity of FM3A cells and transformed cells to G418. Cells were incubated for 6 days with different amounts of G418 and the cell number was determined. ●: FM3A cells without transfection. Transformed cell clone, ○: G-1; △: G-2; ▽: G-3; □: G-4 and ■: G-5.

Fig. 4. Southern blot analysis of FM3A cells and transformants. Lane 1: DNA from FM3A cells without transfection; lanes 2-6: DNAs from transformed cell clones G-1~G-5, respectively.

the agent for 2 months. After this period, the sensitivity of these cell clones, termed G-1~G-5, to G418 was determined. Figure 3 shows the results. Growth of FM3A cells without transfection was completely inhibited at 0.4 mg/ml of G418. All five transformed cell clones showed increased resistance to G418. Transformed cell clones G-1 and G-3 were the most resistant while G-2 and G-5 were the most sensitive to the agent. The results showed that acquired resistance to G418 was stable for at least 2 months of incubation. These cell clones were used to detect plasmid DNA in the cells by Southern blot hybridization.

DNAs were extracted from these transformed cells and digested with restriction endonuclease *Eco*R1, the same enzyme used to linearize pSV2neo DNA before transfection. The membrane containing these DNA blots was hybridized to labeled plasmid DNA. The results are shown in Fig. 4. There was no signal of hybridization in lane 1, which contained FM3A cells without transfection. Lanes 2, 4, and 5, which contained the DNAs of G-1, -3, and -4 cells, showed positive signals of hybridization. As seen from the autoradiogram, the position and intensity of the bands were all different among these cells. A dense band was observed at the position of 5.8 kb with G-1 cells, and less dense bands at 15 kb and 9.6 kb with G-3 and -4 cells, respectively. The different intensities of the bands indicate that the cells contained different numbers of plasmid DNA copies. However, the G-2 and G-5 cells (lanes 3 and 6) did not show positive signals on the autoradiogram.

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