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Gene Transfer: DNA Microinjection Compared with DNA Transfection with a Very High Efficiency

YU-MIN SHEN,[1] RICKY R. HIRSCHHORN,[1] W. EDWARD MERCER,[1] EWA SURMACZ,[2] YOSHIHIRO TSUTSUI,[3] KENNETH J. SOPRANO,[4] AND RENATO BASERGA[1]*

Departments of Microbiology[4] and Pathology and Fels Research Institute,[1] Temple University School of Medicine, Philadelphia, Pennsylvania 19140; Faculty of Biology, Warsaw University, Warsaw, Poland[2]; and Nagoya University, Second Department of Pathology, Nagoya, Japan[3]

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We have developed a procedure that gives a very high efficiency of transfection in mammalian cells with low-molecular-weight DNA (~10^4 base pairs). The procedure uses cells in suspension that are shocked with polyethylene glycol 4 h after replating. We compared this transfection technique to the standard technique involving manual microinjection of DNA into the nuclei of mammalian cells, using recombinant plasmids containing the simian virus 40 A gene or the herpes simplex virus thymidine kinase gene or both. The efficiency of transfection depends on a number of variables, the most important of which is the difference in transfectability of different cell lines. In our laboratory, the cell line that had the highest efficiency of transfection was tk$^-$*ts*13, which is derived from baby hamster kidney cells that are deficient in thymidine kinase and temperature sensitive for growth. Under the appropriate conditions, as many as 70% of these cells can be transfected so that transient gene expression can be detected. With the manual microinjection technique, gene expression is independent of the cell line used and occurs faster than after transfection. The results suggest that the critical stage in transfection is the delivery of DNA molecules to the nucleus. Our experiments also indicate that an enzymatic function, in our case, thymidine kinase activity, gives a higher percentage of positive transfectants than when proteins are visualized only by indirect immunofluorescence. The transfection procedure described in this paper is simple and reproducible and, although less efficient than microinjection, ought to be useful in phenotypic and genotypic studies in which transfer of genes to a large number of cells is desirable.

Manual microinjection of DNA into nuclei of mammalian cells (26) has been successfully used to introduce genes into mammalian cells, where their expression and function can be studied (10, 11). The usefulness of the microinjection technique is limited by the small number of cells that can be microinjected in a reasonable time. An alternative method is the technique of DNA transfection originated by Graham and van der Eb (13) and extensively used in the past few years (6, 15, 16, 18, 25, 30, 36, 39, 40). Transfection is a powerful method for introducing genotypic changes into mammalian cells, but it is less useful in short-term phenotypic studies because of its low efficiency. In our laboratory, we have been interested in the detection of short-term phenotypic changes caused by a variety of genes introduced into mammalian cells. To do this, it was necessary to develop a transfection technique with a reasonably high efficiency. Recently, Chu and Sharp (5) have described a technique in which cells are transfected in suspension. Using simian virus 40 (SV40) DNA and CV-1 cells, they showed that as many as 15 to 20% of cells transfected with SV40 DNA became positive for the SV40 T-antigen. Starting from their procedure, we have investigated different conditions for increasing the efficiency of transfection by cloned DNA fragments. In this paper, we describe in detail a technique with which, under optimal conditions, as many as 70% of the cells become capable of expressing the transfected gene and compare it with the microinjection technique.

MATERIALS AND METHODS

Cell lines and culture conditions. The cell lines *ts*13 (38) and *ts*AF8 (3) are G_1-specific, temperature-sensitive mutants originally isolated from baby hamster kidney cells. The cell line tk$^-$*ts*13 is a thymidine kinase (tk)-deficient mutant of *ts*13 isolated in our laboratory. All of these lines were maintained under culture conditions previously described in detail (1, 10). The mouse cell lines NIH3T3 (obtained from Geoffrey M. Cooper, Sidney Farber Cancer Institute,

Boston, Mass.) and Swiss 3T3 (obtained from Andrea Mastro, The Pennsylvania State University, University Park, Pa.) were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% (vol/vol) fetal calf serum.

The 55-54 cell line is a mouse-human hybrid derived from the fusion of mouse peritoneal macrophages with HT1080 human diploid fibrosarcoma (8). A subclone of the African green monkey kidney cell line CV-1, designated TC-7, was also used in this study. Both cell lines were grown as previously described by Soprano et al. (37).

NG1 cells, an SV40-transformed NIH3T3 cell line, were grown as described by Kawasaki et al. (17). SV3T3 cells are also SV40 transformed but derived from the Swiss 3T3 cells described above.

Plasmids and SV40 DNA. SV40 DNA was isolated by the Hirt procedure (14) from infected CV-1 cells. The recombinant plasmid pSV2G, which contains the entire early region of the SV40 A gene cloned in bacterial plasmid pBR322, was described previously (10, 11). A derivative of pSV2G, designated pSV2Gp$^-$, was constructed by cloning the SV40 fragment of pSV2G into a plasmid obtained from Bethesda Research Laboratories, Rockville, Md. This plasmid contains an insertion of a polydeoxyadenylate-polydeoxythymidylate duplex 100 base pairs in length into the *Pvu*II restriction site of pBR322. As a result of this insertion, it lacks the "poison" sequences of the pBR322 plasmid (21). Recombinant plasmids pC2 and pC6 were constructed by Linnenbach et al. (19) and contain both the entire SV40 genome and the fragment of the herpes simplex virus (HSV) genome that carries the gene for tk.

Plasmid DNA was isolated and purified by phenol extraction and Sepharose 2B chromatography as described by Galanti et al. (11). The purity and structure of all recombinant plasmid DNA were confirmed by restriction analysis as described previously (11).

Transfection procedure. In our experience, small deviations from the protocol considerably affect the efficiency of transfection. For a meaningful comparison with the microinjection technique, it is necessary to describe our novel procedure in detail.

(i) Buffers. (a) Prepare HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid)-buffered saline at 2× strength and store at 4°C in polyethylene tubes until ready to use. 2× HEPES-buffered saline consists of (in grams per liter): HEPES, 10; NaCl, 16; KCl, 0.74; Na_2HPO_4, 7; water, 0.375; dextrose, 2. Use quartz-distilled water. Adjust to pH 7.05 and filter sterilize. (b) Prepare 2 M $CaCl_2$ solution and store in 2-ml samples at −20°C in polyethylene tubes until ready to use. $CaCl_2 \cdot 2H_2O$, 29.4 g/100 ml. Filter sterilize. Do not store for periods longer than 1 month.

(ii) Calcium phosphate-DNA coprecipitate. (a) Prewarm 2× HEPES-buffered saline and 2 M $CaCl_2$ solution to room temperature. (b) Prepare solution A and solution B in 10-ml tubes as follows: solution A, 50 μl of 2 M $CaCl_2$ and 300 μl of sterilized water; solution B, 50 μl of plasmid DNA (1 μg/μl, in sterile water) and 400 μl of 2× HEPES-buffered saline. (c) Blend each solution in a Vortex mixer independently. (d) Using Pipet-aid, bubble air through Pasteur pipette into solution B, and add solution A dropwise very slowly while bubbling; then blend in a Vortex mixer for 15 s at maximal speed. (e) Allow the transfection cocktail to stand at room temperature for 30 to 40 min with periodic agitation. (f) At this point, the transfection cocktail should just be slightly turbid, without visible aggregates. A very fine precipitate can just be visualized under a phase-contrast microscope at ×400.

(iii) Preparation of plating medium. Plating medium was 20.0 ml of conditioned medium from the original cell culture to be transfected plus 17.75 ml of fresh 10% calf serum plus DMEM, 2.0 ml of 2× HeBS, and 0.25 ml of 2 M $CaCl_2$.

(iv) Cell preparation and transfection. (a) Plate cells at a concentration of 2×10^5 cells per dish into 100-mm-diameter petri dishes containing 25 ml of 10% calf serum in high-glucose DMEM and grow for 4 days at 37 or 34°C (for temperature-sensitive mutants); 70 to 80% confluent monolayers are preferable for transfection. (b) On the day of transfection, remove the cells from the subconfluent monolayer, using 0.25% trypsin solution, as follows. (i) Aspirate medium and save it as conditioned medium. Wash monolayer twice with calcium-magnesium-free Hanks solution. (ii) Aspirate Hanks solution and add 5 ml of 0.25% trypsin in Hanks solution; allow it to remain in contact with the cell monolayer until 80 to 90% of the cells have rounded up, as judged by phase-contrast microscopy. (iii) Aspirate the trypsin solution, tap the dish to dislodge the cells, and add 10 ml of 10% calf serum plus DMEM to make cell suspension. (iv) Count the number of cells in a hemacytometer, transfer 1.5×10^6 cells into a 50-ml Corning tube, and then add fresh medium up to 30 ml and wash cells. (v) Centrifuge the cell suspension at a low speed (1,500 rpm, 10 min) at room temperature. (vi) Aspirate the overlaying medium from the cell pellet and leave a small volume of medium (~1.0 ml) in the tube. Then tap the tube to separate the cell pellet. (vii) Add 0.8 ml of transfection cocktail to the cells, pipette them to mix well, and resuspend the cells. (viii) Allow the mixture to stand at room temperature for 20 min with occasional agitation. (ix) Add 36 ml of plating medium and mix by pipetting. (x) Plate 3 ml of the cell suspension onto each of 12 35-mm-diameter petri dishes with 22-mm^2 cover slips. (xi) Incubate at 37 or 34°C for 3 to 4 h.

(v) PEG-sucrose shock. (a) At 3 to 4 h after plating, aspirate the plating medium and add 2 ml of 40% polyethylene glycol (PEG) (Koch-Light, from Research Associates International, Elk Grove, Ill.; molecular weight, 6,000) in 7% (wt/vol) sucrose-DMEM–10 mM $MgCl_2$. (b) After 2 to 3 min, aspirate the PEG solution and wash three times with DMEM (without serum), then add 3 ml of 10% calf serum in DMEM and incubate for 30 to 40 min at 37 or 34°C, and then change to 10% calf serum plus DMEM once more. (c) Incubate at 37 or 34°C for different times, usually 48 to 72 h.

Prepare PEG-sucrose solution as follows. (i) Weigh 10 g of PEG and transfer to a clean 100-ml serum bottle. (ii) Place the bottle in boiling water to melt PEG and add 25 ml of 7% sucrose-DMEM containing 10 mM $MgCl_2$ to the warm PEG solution; mix very well to prevent the PEG from crystallizing. (iii) Allow solution to cool to room temperature; before adding to cells, adjust pH to 7.0 with 1 N HCl.

(vi) Transfection in monolayer. Transfection was carried out as described by Pellicer et al. (30).

Detection of T-antigen by immunofluorescence. SV40 T-antigen was detected by indirect immunofluores-

cence (31) with hamster anti-tumor serum and fluorescein-conjugated goat anti-hamster immunoglobulin G (Cappell Laboratories, Downingtown, Pa.).

Incorporation of [^{3}H]thymidine into tk$^-$*ts*13 cells. Thymidine kinase activity was detected in tk$^-$*ts*13 cells by continuous labeling with [^{3}H]thymidine (6.7 Ci/mmol, 0.7 μCi/ml; New England Nuclear Corp., Boston, Mass.), as indicated in Results. Autoradiographs were prepared and analyzed by standard methods.

Manual microinjection procedure. Plasmid DNA was injected directly into the nuclei of cells with the glass capillary microinjection method of Graessmann and Graessmann (12). Samples were dissolved in 10 mM Tris-hydrochloride (pH 7.5) at 25°C and routinely centrifuged for 15 min in an Eppendorf centrifuge before microinjection.

Preparation of spheroplasts. Bacterial spheroplasts were prepared from *Escherichia coli* LE392 containing recombinant plasmid pSV2G and were fused to mammalian cells as described by Schaffner (32).

Selection of transformants. Mouse 3T3 transformants were selected in soft agar after transfection with SV40 DNA or with pSV2G DNA. DNA-treated cells were transferred to semi-solid DMEM containing 0.3% agar (Difco) over a preformed layer of medium containing 0.5% agar prepared as described by MacPherson and Montagnier (22) 6 days after transfection. Colonies of transformed cells (2 mm in diameter) were counted after 2 to 3 weeks of incubation, removed from the agar, and expanded before testing for their expression of T-antigen by indirect immunofluorescence.

Hamster tk$^-$*ts*13 transformants were selected as follows. About 72 h after transfection with pC6 DNA, tk$^-$*ts*13 cells were trypsinized and replated in a mixture of (in micrograms per milliliter): glycine, 0.23; hypoxanthine, 13.6; aminopterin, 0.19; and thymidine, 3.8 (GHAT) containing 10% calf serum in a 100-mm culture plate at a density of 10^6. After 11 days in selective medium, one plate was stained with Giemsa, and colonies were isolated from other plates and replated individually in 16-mm tissue culture plates. These clones were expanded and then grown in GHAT medium containing 2% calf serum and tested for their ability to express T-antigen by indirect immunofluorescence.

Immunoprecipitation methods for the detection of T-antigen in transfected cells. tk$^-$*ts*13 cells were transfected in suspension as described above, with the following modification. About 0.8×10^6 transfected cells were plated in 100-mm culture plates, and the remaining cells were plated in several 35-mm culture plates with 22-mm^2 cover slips to determine the efficiency of transfection by immunofluorescence. After incubation at 34°C for 4 h, these cells were subjected to 70% PEG shock (7 g of PEG plus 10 ml of 7% [wt/vol] sucrose–10 mM $MgCl_2$ in DMEM). At 22 h after transfection, the cells were rinsed with methionine-free DMEM containing 10% nondialyzed calf serum, and then 4 ml of this medium containing 100 μCi of [^{35}S]methionine (962 Ci/mmol; New England Nuclear Corp.) was added. At 48 h after transfection, the cultures were rinsed five times with ice-cold phosphate-buffered saline. After carefully aspirating all of the phosphate-buffered saline, the monolayer was covered with 1.5 ml of ice-cold extraction buffer per plate (0.1 M Tris-hydrochloride [pH 9.0]–0.1 M NaCl–0.005 M KCl–0.001 M $CaCl_2$–0.0005 M $MgCl_2$–0.5% [wt/wt] Nonidet P-40–1 mM phenylmethylsulfonyl fluoride), and immunoprecipitation and electrophoresis were carried out as described by Schwyzer and co-workers (34).

RESULTS

Transfection efficiency of SV40 DNA versus a cloned fragment of SV40 containing the entire early A gene region. African green monkey kidney cells (TC-7) were transfected in suspension with either SV40 DNA or DNA from the recombinant plasmid pSV2G, which contains the entire SV40 A gene fragment cloned into the bacterial plasmid pBR322 (11). Figure 1 shows the time course of T-antigen expression in TC-7 cells. Transfection with SV40 DNA was more efficient than transfection with pSV2G, even though equivalent amounts of DNA (50 μg/ml) were used (Fig. 1). At 24 h posttransfection, 20% of the TC-7 cells transfected with SV40 DNA were T-antigen positive by indirect immunofluorescence, whereas with pSV2G the percentage was only 8%. Furthermore, when pSV2G DNA was used, the peak of T-antigen-positive cells reached a maximum of 10% at 40 h and then declined sharply thereafter. In contrast, when SV40 DNA was used, the percentage of cells that expressed T-antigen continued to increase, and by 73 h it was approximately 40%. We attribute this to the fact that TC-7 cells are permissive for SV40 DNA replication, and the high percentage of cells that express T-antigen at 73 h after exposure to SV40 DNA may represent secondary infection by SV40 virions.

Therefore, any attempt to determine the efficiency of transfection should take into account the fact that with SV40 DNA and permissive cells, such as TC-7 cells, the percentage of T-antigen-positive cells may be artificially high, owing to secondary infection of surrounding cells. On the other hand, the sharp decline in T-

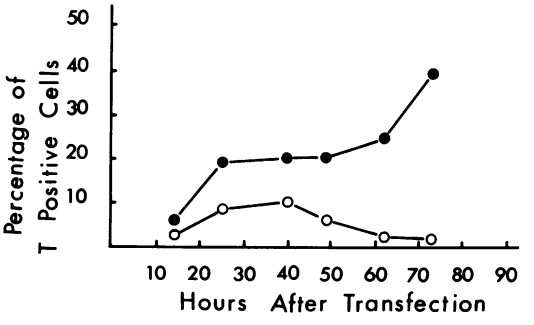

FIG. 1. Percentage of cells positive for the SV40 T-antigen after transfection with either SV40 DNA (●) or pSV2G DNA (○). TC-7 cells were transfected in suspension as described in the text. SV40 T-antigen was detected by indirect immunofluorescence.

TABLE 1. Efficiency of DNA transfection with cells in suspension or in monolayer[a]

Cell line	% T-positive cells in:	
	Suspension	Monolayer
Swiss 3T3	5.2–6.3	0.1–0.3
NIH3T3	2.6–3.1	0.2–0.6
tk⁻*ts*13	16.0–22.0	0.5–0.2

[a] All cells were transfected with pSV2G DNA and fixed for immunofluorescence 48 h later. Transfection in suspension and in monolayer and the technique for indirect immunofluorescence were carried out as described in the text. The numbers are ranges from separate experiments.

antigen-positive cells after 40 h when pSV2G DNA is used could be explained by the fact that recombinant plasmids propagated in *E. coli* replicate poorly, if at all, in cells of simian origin after transfection (21, 28).

Comparison of transfection of cells in suspension versus cells in monolayers. Transfection of cells in suspension is more efficient than transfection in monolayers. Our results (Table 1) indicate that with both tk⁻*ts*13 and 3T3 cells, transfection of cells in suspension with pSV2G DNA is several times more efficient than transfection of cells in monolayers.

Efficiency of transfection in different cell lines with plasmid DNA. In these experiments, several cell lines available in our laboratory and listed in Materials and Methods were transfected by the suspension technique with either pSV2G or pC2 DNA. The appearance of T-antigen or the ability of cells to incorporate [^{3}H]thymidine was determined after transfection (Table 2). Notice that tk⁻*ts*13 cells were the most efficiently transfected. With pC2 DNA, as many as 34% of the cells became T positive and as many as 58% became capable of incorporating [^{3}H]thymidine (in short-term experiments like this, the background of the control tk⁻*ts*13 cells is 0, since these cells do not incorporate [^{3}H]thymidine at all). Notice also that *ts*AF8 cells, which, like tk⁻*ts*13 cells, are derived from baby hamster kidney cells (24), were highly transfectable by pSV2G or by pC2. 55-54 cells and TC-7 cells also yielded a reasonable percentage of cells that express the T-antigen. 3T3 cells were the most difficult to transfect, the percentage of T-positive cells being about 3%. Clearly, the type of cell line is important in determining the efficiency of transfection.

Efficiency of microinjection is the same in different cell lines. The same cell lines were tested for their ability to express SV40 T-antigen when manually microinjected with pSV2G (Table 2). All cell lines expressed T-antigen efficiently after microinjection. As already reported by Galanti et al. (11), pSV2G DNA must be supercoiled or, at most, nicked for an efficient microinjection. Linear DNA was considerably less active and totally inactive if microinjected into the cytoplasm. Incidentally, linear pSV2G DNA was also inactive in transfection (data not shown).

The efficiency of transfection did not depend on the ability of the transfected gene to be expressed differently in different cell lines (Table 2). When the same gene was directly delivered into the nuclei, expression was high in all cell lines.

Time course of gene expression after transfection of cells in suspension. Figure 2A shows the time course of expression of T-antigen in different cell lines transfected with pSV2G DNA. In two cases, *ts*AF8 and *ts*13 cells, the maximum expression of T-antigen was reached at 72 h after transfection. The maximum expression of tk⁻*ts*13 and 55-54 cells was reached earlier, at 48 h, and decreased thereafter.

TABLE 2. Efficiency of DNA transfection or microinjection in several cell lines

Cell line	Transfection with[a]:			Microinjection with pSV2G (% T^+ cells)[b]
	pSV2G (% T^+ cells)	pC2		
		% T^+ cells	% Labeled cells	
tk⁻*ts*13	25.2	33.7	58.1	63.8
*ts*AF8	21.5	8.2	—	60.1
*ts*13	9.7	14.5	—	85.0
TC-7	8.2	4.8	—	92.0
55-54	6.9	7.6	—	79.4
NIH3T3	3.0	<1.0	—	73.8

[a] All cells were transfected in suspension with either pSV2G or pC2 DNA and were fixed 48 h after transfection. % T^+ cells, Cells positive for SV40 T-antigen by indirect immunofluorescence; % Labeled cells, cells capable of incorporating [^{3}H]thymidine.

[b] All cells were microinjected with pSV2G at a concentration of 1 mg of DNA per ml and fixed 24 h later. The microinjection technique used was as described by Graessmann and Graessmann (12). Percentages given are the best results obtained for each cell line over a period of several months. However, the figures are very close to average figures. For instance, with TC-7 cells, the percentage of T-positive cells was above 80% in 15 of 16 trials.

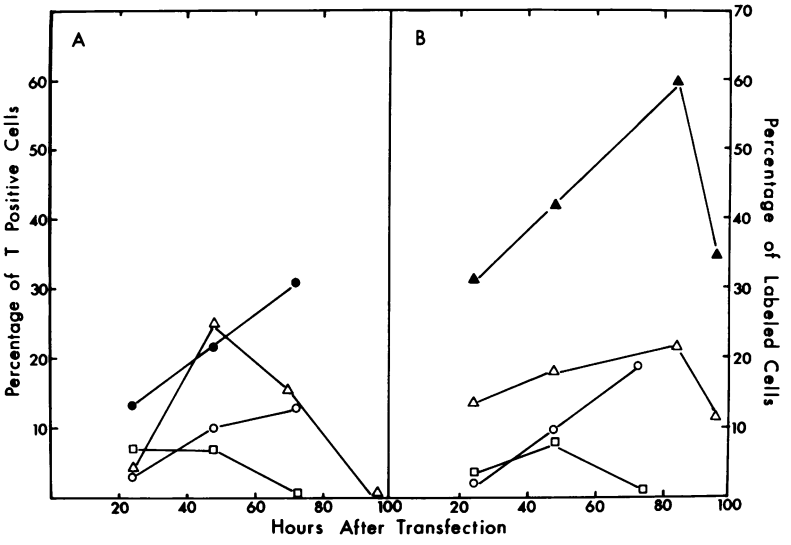

FIG. 2. (A) Percentage of cells positive for SV40 T-antigen at various times after transfection with pSV2G DNA. ●, *ts*AF8 cells; ○, *ts*13 cells; □, 55-54 cells; △, tk$^-$*ts*13 cells. Cells were transfected in suspension as described in the text. SV40 T-antigen was detected by indirect immunofluorescence. (B) Efficiency of transfection with pC2 plasmid DNA. △, Percentage of tk$^-$*ts*13 cells capable of incorporating [^{3}H]thymidine. All other symbols represent percentages of cells positive for SV40 T-antigen by indirect immunofluorescence: △, tk$^-$*ts*13 cells; ○, *ts*13 cells; □, 55-54 cells.

Figure 2B shows the time course of gene expression after transfection of cells in suspension with pC2 DNA. The same cell lines, except for *ts*AF8, were used. In addition, the ability of tk$^-$*ts*13 cells to incorporate [^{3}H]thymidine is shown. The peak of gene expression was generally around 72 h and decreased afterwards. 55-54 cells were an exception, reaching a peak at 48 h. Notice that in tk$^-$*ts*13 cells transfected with pC2 DNA, the percentage of cells capable of incorporating [^{3}H]thymidine was consistently higher than the percentage of cells that became T positive. We shall return to this later.

This time course of T-antigen expression obtained by transfection is different from that obtained by microinjection (Fig. 3). With microinjection of pSV2G, maximum expression was reached within 24 h, when, because of cell

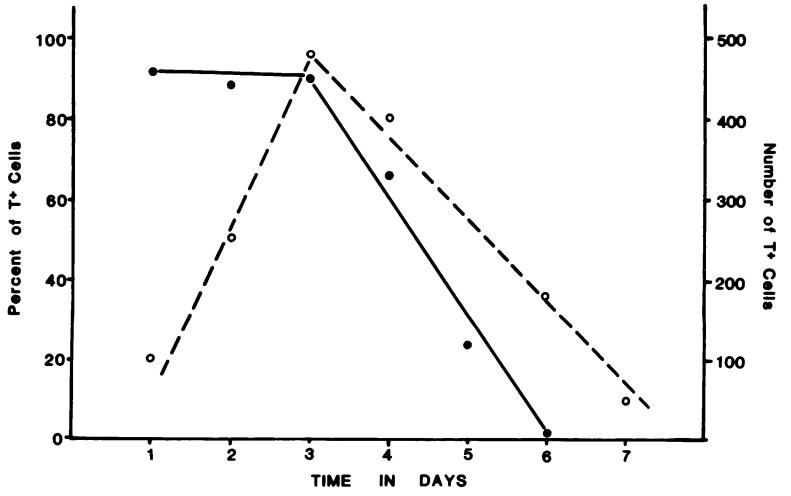

FIG. 3. Time course of T-antigen expression in *ts*13 cells microinjected with pSV2G DNA. A total of 100 cells in a closed circle were microinjected, and T-antigen expression was determined by indirect immunofluorescence. ●, Percentage of T-positive cells; ○, absolute number of T-positive cells in the circle. Results of two separate experiments are shown, which may account for slight discrepancies between the two curves.

TABLE 3. Effect of different concentrations of PEG on the efficiency of DNA transfection in tk$^-$*ts*13 cells[a]

PEG concn (%)	DNA transfection in tk$^-$*ts*13 cells with:			Transfection in TC-7 cells with pSV2G (% T$^+$ cells)
	pSV2G (% T$^+$ cells)	pC2		
		% T$^+$ cells	% Labeled cells	
0	2.3	7.2	14.2	5.0
30	6.7	15.4	39.3	—
40	10.0	24.4	50.2	11.7
50	14.6	23.1	50.3	—
60	27.4	28.6	71.6	—
70	46.1	36.3	67.8	—
Glycerol	—	—	—	3.2

[a] Cells were transfected in suspension. 30% PEG, Three grams of PEG (molecular weight, 6,000) plus 10 ml of DMEM. % T$^+$ cells, Cells positive for SV40 T-antigen by indirect immunofluorescence; % Labeled cells, cells capable of incorporating [^{3}H]thymidine. Glycerol was used as a 15% solution. Cells were harvested 48 h after transfection. Other tests were performed as described in Tables 1 and 2.—, Not done.

proliferation, the number of T-positive cells actually increased, stayed high for 2 more days, and then dropped sharply.

In similar experiments, we investigated whether pSV2G p$^-$ would be more effective than pSV2G in the efficiency of transfection. pSV2G p$^-$ is the same as pSV2G, except that the so-called poison sequences of the pBR322 have been mutated by insertion. Supposedly, this should allow the plasmid to replicate more freely in mammalian cells (21). In our hands, pSV2G p$^-$ was no more efficient in its ability to transfect cells than the standard pSV2G. With *ts*AF8, 10.5% of the cells were T positive at 48 h and 18.3% were T positive at 72 h, which is not better than the percentage (21%) obtained with pSV2G. Furthermore, transfection of *ts*13 cells with pSV2G p$^-$ yielded a percentage of T-positive cells of about 1%, less than that (9.7%) obtained with pSV2G.

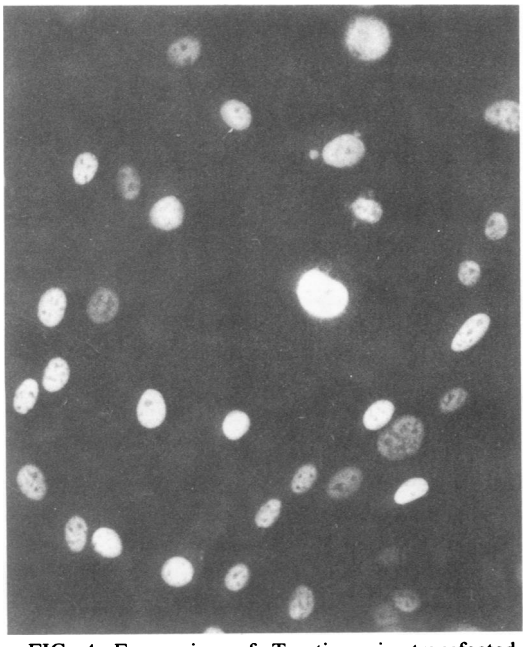

FIG. 4. Expression of T-antigen in transfected cells. tk$^-$*ts*13 cells were transfected with pSV2G DNA, using a 70% PEG-sucrose shock. After 48 h, the cells were fixed and stained by indirect immunofluorescence with anti-T antibody. The methods used were as described in the text.

Effect of different concentrations of PEG on the efficiency of transfection of tk$^-$*ts*13 cells. To determine the effect of different PEG concentrations on the transfection efficiency of tk$^-$*ts*13 cells, we carried out transfection on tk$^-$*ts*13 cells in suspension as described in Materials and Methods, except the concentration of PEG used for shocking the cells varied. The results for both pSV2G and pC2 are shown in Table 3. Clearly, as the concentration of PEG increased, the percentage of cells that became T positive with either pSV2G or pC2 increased, reaching a maximum of 45% at a PEG concentration of 70%. At this concentration of PEG, expression of the tk gene reaches 70%. Representative fields are shown in Fig. 4 and 5. Although these results were quite clear-cut with tk$^-$*ts*13 cells, variations in PEG concentrations did not necessarily improve transfection in other cell lines. For instance, PEG shock has very little effect on the percentage of T-positive cells in 55-54 cells, the percentage ranging from 4.9% with no shock at all to 6.9% with a 3-min, 40% PEG shock. The length of exposure to PEG was also critical. In general, 2 min of exposure was optimal, although with some cells, we obtained good results even with a 4-min exposure. Longer times resulted in toxicity to the cells (data not shown). Finally, notice that with TC-7 cells PEG was four times more effective than 15% glycerol in inducing T-antigen expression. In general, PEG shock was useful in most cases, but the proper concentration of PEG must be worked out for each individual cell line or strain.

Expression of T-antigen and of the tk gene in

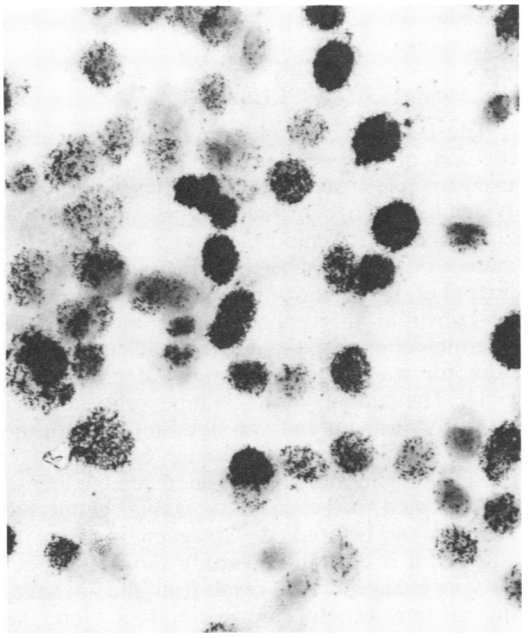

FIG. 5. Expression of the tk phenotype on transfected cells. tk$^-$*ts*13 cells were transfected with pC2 DNA, using a 60% PEG-sucrose shock. Cells were labeled from 24 to 48 h later with [^{3}H]thymidine and then autoradiographed to detect tk activity. The methods used were as described in the text.

cells transfected or microinjected with recombinant plasmids. As noted before, the percentage of tk$^-$*ts*13 cells capable of incorporating [^{3}H]thymidine was consistently higher (usually double) than the percentage of cells that were T positive when these cells were transfected with pC2, which contains both the SV40 genome and the tk gene of HSV. Table 4 shows another example of the consistent difference in detection of gene expression of these two genes when they are introduced by transfection. Table 4 also shows that this is also true when cells are microinjected with either pC2 or comicroinjected with pSV2G and with the HSV tk clone. In all cases, the percentage of tk$^-$*ts*13 cells capable of incorporating [^{3}H]thymidine was higher than the percentage of cells that became T positive.

This was not owing to metabolic cooperation, since cocultivation of *ts*13 cells with tk$^-$*ts*13 cells did not result in the latter becoming capable of incorporating [^{3}H]thymidine (data not shown).

Immunoprecipitates of cells transfected with pSV2G. Figure 6 shows immunoprecipitates of tk$^-$*ts*13 cells transfected with pSV2G in which the proteins labeled with [^{35}S]methionine were immunoprecipitated with anti-T antibody as described in Materials and Methods. Notice that the transfected cells produced a protein that was specifically precipitated by anti-T antibody. This protein did not seem to be different in size, based on its migration on gels, from the T-antigen that appeared in cells infected with SV40. We were not able to detect a visible band of T-antigen by immunoprecipitation in TC-7 cells microinjected with pSV2G, even when 3,000 cells were microinjected.

Conditions that fail to improve transfection of cells in suspension. Several conditions tried by us failed to improve the efficiency of transfection of tk$^-$*ts*13 cells in suspension. All of these various conditions had a rationale in the literature for being tried, but all of them completely failed to increase the efficiency of transfection. These included amphotericin B (2), cycloheximide, which increases production of mRNA in adenovirus-infected cells (7), 12-O-tetradecanoyl phorbol 13-acetate (TPA), which is known to induce replication of episomal Epstein-Barr virus DNA (27), DEAE dextran, which increases transfection with SV40 DNA (23), calcium starvation, which improves fusion (33), and poly-L-lysine-coated cover slips as well as spheroplasts that, in our hands, never gave results better than 3 to 4% T-positive cells. We repeated the experiments with spheroplasts several times and, at least in our hands, the spheroplasts were very toxic. The maximum number of TC-7 cells that became T positive never went above 4% and, if the number of spheroplasts was increased, marked cell death would ensue. The high incidence of cell death may account for some higher percentages of T-positive cells reported by other investigators, since the few surviving cells were more efficiently fused. It should also be remembered that dying cells become immunofluorescent and can sometimes be mistaken for T-positive cells. That the spheroplasts may contain some toxic material for mammalian cells was indirectly confirmed by the fact that, when plasmid DNA is microinjected, we noticed that all bacterial DNA must be carefully removed, or the microinjected DNA will be toxic (data not shown).

TABLE 4. Expression of genes introduced into tk$^-$*ts*13 cells[a]

Method	% Cells		
	T$^+$	Labeled	tk$^+$
Microinjection	61.7	97.1	47
Transfection	18.0	42.4	ND[b]

[a] T$^+$ cells, Cells stainable for SV40 T-antigen; labeled cells, cells capable of incorporating [^{3}H]thymidine; tk$^+$ cells, cells stainable with a specific antibody against HSV tk (kindly provided by Saul Kit). The genes introduced into cells were the SV40 A gene and the HSV tk gene, cloned in pBR322, either as pC2 plasmid or comicroinjected as pSV2G plus pTK.

[b] ND, Not done.

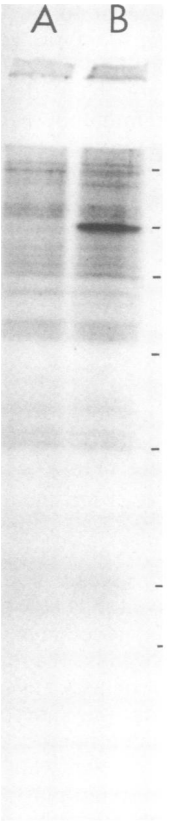

FIG. 6. Immunoprecipitate of cells transfected with pSV2G DNA. tk$^-$*ts*13 cells were transfected with pSV2G DNA, using a 70% PEG-sucrose shock, and labeled with [^{35}S]methionine. Cells were extracted and immunoprecipitated, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. The gel was treated with En^{3}Hance, dried, and fluorographed. Lane A, Cell extract precipitated with normal hamster serum, lane B, cell extract precipitated with anti-T serum. The position of simultaneously run molecular-weight markers are indicated by horizontal lines, and are, from top to bottom: myosin (H-chain), 200,000; phosphorylase B, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lacto globulin, 18,400; cytochrome *c*, 12,300.

Transformation of cells by either pSV2G or pC6. Table 5 shows the efficiency of transformation in Swiss 3T3 cells transfected with either pSV2G DNA or in tk$^-$*ts*13 cells transfected with pC6. In both cases, an efficiency close to 20 to 30 transformants per 10^5 cells was obtained. The 3T3 cells transformed with pSV2G were selected by soft agar cloning, whereas the tk$^-$*ts*13 cells transfected with pC6 were selected in GHAT medium. After expansion, the clones were tested for their ability to express the T-antigen by indirect immunofluorescence. More than half of the GHAT-selected clones were not only expressing the tk gene, but were also positive for T-antigen.

DISCUSSION

The DNA calcium-phosphate coprecipitation method of transfection has been extensively used to investigate both phenotypic and genotypic changes in cultured mammalian cells. As a consequence, its importance as a research tool cannot be overemphasized (for review, see Pellicer et al. [29]). The purpose of the experiments described in this paper was to compare the microinjection technique with an efficient procedure for transfecting a variety of mammalian cells. The monolayer technique originally devised by Graham and van der Eb (13) with the recent modifications of Wigler et al. (39) is usually sufficient to yield an adequate number of transformed cells so that stable genotypic changes can be studied. However, in some instances, it is desirable to study short-term phenotypic changes which result from the introduction of new genetic information. As such, the transfection technique described in this paper, which is essentially a modification of the procedure described by Chu and Sharp (5), seems to be a reasonable alternative to the manual microinjection technique (12) and gives excellent results in terms of transient gene expression (phenotypic as opposed to genotypic changes).

Manual microinjection is definitely more efficient than transfection, even with the modifications described in this report, but it is, of course, a time-consuming and delicate technique in which only a limited number of cells can be microinjected.

From the experiments described above, the following conclusions can be drawn. (i) Transfection of cells in suspension, as suggested by Chu and Sharp (5), is definitely more efficient than transfection of cells in monolayers. (ii) The efficiency obtained by Chu and Sharp (5) for

TABLE 5. Transformation efficiency after transfection of cells in suspension[a]

Transfected cells	DNA	No. of clones in selective medium per 10^6 cells
tk$^-$*ts*13	pC6	>300
Swiss 3T3	pSV2G	180–250

[a] Transfection and selection were carried out as described in the text. Cells transfected with pC6 were selected in GHAT medium. With tk$^-$*ts*13 cells, at least 300 colonies were counted. Because they were so close to each other, it is very likely that some of them consisted of two or more clones. For this reason, the number of clones is given as >300, since a precise number cannot be given.

transfection with SV40 DNA of CV-1 cells was, however, probably artificially high because of reinfection of permissive cells by SV40 virions. The true efficiency can only be determined with a noninfective SV40 genome. (iii) The most important finding is that the efficiency of transfection of cells in suspension depends very much on the cell line used, as already suggested by the experiments of Corsaro and Pearson (6), with subclones of LTK$^-$ cells. In other words, different cell lines have different transfectability, and from our results (Table 2), it seems that cells derived from the BHK line have, in general, higher transfectability than other cell lines. tk$^-$*ts*13 cells turned out to be the best of all. These cells are particularly attractive for transfection experiments because they are temperature-sensitive for growth (9, 38), tk$^-$, and can be permanently transformed quite easily. This cell line is now available to all qualified investigators upon written request. (iv) The PEG-sucrose shock is a definite improvement on the technique used by Chu and Sharp (5); especially when the PEG shock is done with a 60 to 70% concentration of PEG, the efficiency of transfection, at least for some cells, is very high. (v) By both transfection experiments and manual microinjection experiments, it is easier to detect certain enzymatic functions than to detect proteins by immunofluorescence. This is, of course, not surprising. Presumably, the number of molecules necessary to give a detectable immunofluorescent-positive test is higher than that required to carry out an enzymatic function. This is also true of the tk gene itself. When the tk gene is microinjected into tk$^-$*ts*13 cells, the percentage of cells capable of incorporating [^{3}H]thymidine is consistently higher than the percentage of cells that become immunofluorescent after staining with an antibody specific for the HSV tk gene. In evaluating the efficiency of transfection one should, therefore, keep in mind that immunofluorescence probably gives a lower value. (vi) From these experiments and from experiments with manual microinjection, we propose as a hypothesis that the rate-limiting step is the number of DNA molecules that actually enter the nucleus, as already proposed by Loyter et al. (20). For instance, the efficiency of gene expression is better when the genes are manually microinjected into the nucleus than when they are microinjected into the cytoplasm (4, 11). Indeed, if the DNA that is microinjected into the cytoplasm is linear, the efficiency of gene expression drops dramatically, presumably because linear DNA is more rapidly degraded than circular DNA. More important, the efficiency of gene expression is very much the same in different cell lines when the gene is microinjected, whereas it varies greatly when the gene is transfected. This can be interpreted as indicating that the rate-limiting step is the number of gene copies arriving in the nucleus.

We theorized that the real barrier to the expression of genes introduced by transfection into mammalian cells is actually the nuclear membrane, and for this reason we have tried to transfect cells in suspension that have been synchronized in the mitotic phase of the cell cycle. Unfortunately, since the transfection cocktail contained a considerable amount of calcium, the mitotic cells promptly clumped as soon as they were exposed to the transfection cocktail and would not replate (unpublished data). However, Sene and Nicolau (35) have been able to considerably increase transfection through liposomes by using cells synchronized in the mitotic phase of the cell cycle. According to Sene and Nicolau (35), as many as 75% of the cells can be efficiently transfected with gene-containing liposomes if they are in mitosis. This seems to indicate that the nuclear membrane is the actual critical barrier to the efficient penetration of transfected genes into the nucleus.

Whatever is the rate-limiting step in the efficiency of transfection, the procedure described here is simple and reproducible and compares favorably with the microinjection technique if one desires to introduce genes into a large number of viable mammalian cells to detect their expression.

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