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Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading

(gene expression)

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ABSTRACT **Scrape loading and sonication loading are two recently described methods of introducing macromolecules into living cells. We have tested the efficacy of these methods for transfection of mammalian cells with exogenous DNA, using selection systems based either on resistance to the drug G418 (Geneticin) or on acquisition of the ability to utilize the salvage pathway of pyrimidine biosynthesis. These loading methods can be employed to generate cell lines that express the gene product of the transfected DNA molecules both transiently and stably. Optimal transfection is observed when the DNA is added to cells in physiological saline lacking divalent cations and containing K^+ in place of Na^+. DNA molecules 7.1 to 30 kilobases long have been introduced by the scrape loading procedure. In addition, the scrape loading procedure has been employed for cotransfection and subsequent expression of nonselectable genes encoded on DNA molecules added in a mixture with DNA molecules whose expression is selected. Cell lines expressing oncogenes or proteins that are important for regulation of cell growth and division have been obtained by this procedure. The scrape loading procedure is also useful for studies of the cellular changes that occur upon expression of an exogenous gene. As many as 80% of cells scrape loaded with the plasmid pC6, which encodes the simian virus 40 large tumor antigen, contained this protein in the nucleus between 1 and 5 days after transfection. Thus, scrape loading and sonication loading are simple, economical, and reproducible methods for introduction of DNA molecules into adherent and nonadherent cells, and these methods may be useful in the future for experimentation at both fundamental and applied levels.**

Methods for introduction of foreign DNA into living cells in a form allowing its expression in the recipient cell have been widely used in development of human gene therapy technologies (1), in strategies for plant genetic engineering (2), in improvement of domestic livestock (3, 4), and in basic research. A number of methods for introduction of DNA into living cells are currently in use (5). These techniques include direct microinjection (6, 7), precipitation with calcium phosphate (8, 9), membrane breakdown induced by transient electric fields (10–12), fusion of erythrocytes or liposomes (13–16), osmotic lysis of pinosomes (17), centrifugation loading (18), delivery of high-velocity microprojectiles (19), retroviral vectors (20, 21), and the natural plant vector *Agrobacterium tumefaciens* (22).

Two additional methods for introduction of macromolecules into living cells, scrape loading (23) and sonication loading (24), have recently been described. In this report, we describe the application of scrape loading and sonication loading to the introduction of functional genetic material into living cells, and we discuss the potential utility of these methods as supplements to the existing arsenal of methods for obtaining cellular expression of sequence information present on molecules of exogenous DNA.

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MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, penicillin, streptomycin, Geneticin (G418 sulfate), and Fungizone were supplied by GIBCO. Hypoxanthine, aminopterin, thymidine, and fluorescein-labeled dextran (FD-40) were obtained from Sigma. Murine fibroblasts deficient in the enzyme thymidine kinase (LTK^-) and the pPVTK4 plasmid, which contains the thymidine kinase gene in pBR328, were kindly supplied by Peter Stambrook (University of Cincinnati). Plasmids were grown in *Escherichia coli* and the DNA was purified by cesium chloride gradient centrifugation in the presence of ethidium bromide as described (25). Adenovirus 2 DNA and pSV2neo (26) were purchased from Bethesda Research Laboratories. HeLa cells, monoclonal antibody (PAB101) reactive with the simian virus 40 (SV40) large tumor (T) antigen (TIB117), and c-Ha-*ras* DNA were purchased from the American Type Culture Collection. A 6.6-kilobase (kb) *Bam*HI fragment containing the *ras* oncogene was inserted into the *Bam*HI site of pBR322. Plasmid pC6, which encodes the SV40 large T antigen, was obtained from Yakov Gluzman (Cold Spring Harbor Laboratories). The insert was at the *Eco*RI site of pMKI6 #6. Rat embryo fibroblasts (REF) were produced as described (27) and WT/O-15.5 D2 (abbreviated D2) cells were obtained from Paul Fisher (Columbia University). The murine myeloma cell line P3-X63-Ag8.653 (28) was provided by Dan Murfin (University of Georgia).

Cell Growth. HeLa cells, hepatic tissue culture cells, and LTK^- fibroblasts were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, penicillin at 50 units/ml, streptomycin at 50 μg/ml, and Fungizone at 2.5 μg/ml (complete medium). The murine myeloma P3-X63-Ag8.653 was grown in the same medium containing 50 μM 2-mercaptoethanol. REF and D2 cells were grown in plastic tissue culture flasks (Corning), using Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, penicillin at 50 units/ml, streptomycin at 10 μg/ml, 0.3% sodium bicarbonate, 1 mM glutamine, and 2.5 mM Hepes.

Introduction of Thymidine Kinase Gene into LTK^- Fibroblasts by Scrape Loading and Sonication Loading. In the scrape loading procedure, culture medium was removed from a 10-cm Petri dish with a nearly confluent monolayer of LTK^- cells. The cell monolayer was then washed once with

Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; REF, rat embryo fibroblasts.

5 ml of 10 mM KH_2PO_4/1.0 mg of glucose per ml/0.1 mM EDTA/140 mM NaCl, pH 7.2 (loading solution). pPVTK4 DNA (0–50 μg/ml) in 1 ml of the loading solution was added to the cells. The cells were then loaded with DNA by scraping them from the dish by using a rubber policeman as previously described (23). Cells were counted by using a hemocytometer, and viable cells were identified by exclusion of the dye trypan blue. In some experiments, variations in the loading solution were employed in which KCl was used in place of NaCl and in which the solutions were supplemented with 2 mM $MgCl_2$.

In the sonication loading procedure, after removal of medium from a nearly confluent monolayer of LTK^- fibroblasts in a 10-cm dish, the cells were washed with 5 ml of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and removed from the substrate by trypsin digestion. The cells were diluted into 2 ml of complete medium, sedimented, resuspended in 10 ml of loading solution, and resedimented. The cells were then suspended in 1 ml of loading solution containing pPVTK4 DNA (0–50 μg/ml) in a sterile 12 × 75 mm plastic round-bottom tube and sonicated with three bursts of 0.5-sec duration at power setting 1 of a Branson model 200 sonicator equipped with a two-step tapered microtip fitted with a plastic sleeve. This sonication loading method is an adaptation of a technique developed for use with *Dictyostelium discoideum* (24, 29). Trial experiments indicated that the viability of LTK^- fibroblasts under these conditions is approximately 70%. The number of viable cells was determined by counting in the presence of trypan blue.

One million viable cells loaded with pPVTK4 DNA either by scraping or by sonication were diluted into 10 ml of complete culture medium supplemented with 100 μM hypoxanthine and 16 μM thymidine (HT medium) in a 10-cm culture dish. Three days later, 0.4 μM aminopterin was added to the plates to initiate selection against cells lacking thymidine kinase. Fresh medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) was added 1 week after initiating the selection. After an additional 2 weeks of growth, the plates were washed, fixed, and treated with Giemsa stain to allow macroscopic enumeration of colonies as previously described (27). In some cases, colonies were subcloned by trypsin digestion in cloning rings and grown in mass culture in HAT medium to demonstrate the stability of the transfection.

Transformation of REF and D2 Cells with pSV2neo by Scrape Loading. REF or D2 cells were grown in 10-cm Petri dishes to 80% confluency. The medium was removed, and 0.2 ml of a solution containing 1 or 2 μg of pSV2neo DNA in PBS was spread over the cells and allowed to sit for 5 min. The cells were then scraped from the dish, allowed to sit for 10 min, suspended in 10 ml of medium buffered with 25 mM Hepes, and distributed into fresh culture vessels at a ratio of 1:2. Three days later, selection was initiated by addition of Geneticin (G418 sulfate) at 200 μg/ml. After 2 weeks, cells in control dishes were dead, and foci of about 10–15 cells had formed in the other dishes. Foci were picked by using cloning rings, transferred to 96-well microtiter plates for 1 week, transferred to 12-well plates until established, and then transferred to 25-mm tissue culture flasks for mass culture. Geneticin was omitted during mass culture.

Cotransfection of REF and D2 cells was performed by mixing pSV2neo DNA and either plasmid PC6 (SV40 large T antigen), wild-type adenovirus 2 genome, or *ras* T24 oncogene before addition to the cells. DNA was mixed in a final volume of 0.2 ml of PBS and contained a ratio of pSV2neo DNA to the cotransfected DNA of 1:10. Transfection, selection, and culture were performed as described above.

Quantification of Loading Efficiency by Using Fluorescein-Labeled Dextran. Cells were loaded with fluorescein-labeled dextran by using either the scraping or the sonication protocol. Fluorescein-labeled dextran was added to cells at a final concentration of 10 mg/ml, and cells were then treated as described above. Cells exhibiting cytoplasmic fluorescence were enumerated by examination of the living cells by fluorescence microscopy both 6 and 24 hr after loading. Control experiments included removal of cells from the substrate by trypsin digestion in the presence of fluorescein-labeled dextran and addition of the fluorescein-labeled dextran to the cells after scraping or sonication treatments had been performed. Little or no loading was observed in these control experiments.

Quantification of Transient Expression by Immunofluorescence Microscopy. REF cells were loaded by scraping with 10 μg of plasmid pC6 DNA (SV40 large T antigen) in 0.2 ml of PBS. Scraped cells were plated in four-well chamber slides, fixed 0, 1, 2, 3, 4, or 5 days later, and stained with antibody reactive with the SV40 large T antigen to detect expression of the transformed gene product as described (30), except methanol was used as the fixative agent. Positive cells exhibited bright nuclear fluorescence characteristic of the large T antigen in SV40-infected cells.

RESULTS

Use of Scrape Loading and Sonication Loading for Transfection of Mammalian Cells. Scrape loading (23) and sonication loading (24) have both been employed previously for introduction of macromolecules such as fluorescently-labeled dextran and protein molecules into the cytoplasm of living eukaryotic cells. Previous use of the sonication loading method has, however, been restricted to experiments with amoebae of the cellular slime mold *D. discoideum*. We have tested the efficacy of these techniques for transformation of mammalian cells with DNA.

The plasmid pPVTK4, which contains the herpes virus thymidine kinase gene in pBR328, has been used to transfect LTK^- fibroblasts, which lack this enzyme. LTK^- cells are good recipients for both scrape and sonication loading as assessed by introduction of fluorescein-labeled dextran having a molecular mass of 40,000 daltons (Table 1). Loading with fluorescein-labeled dextran at 10 mg/ml by either scraping or sonication resulted in loading of 50% and 20% of LTK^- cells, respectively, as assessed by observations of living cells by fluorescence microscopy (Fig. 1 and Table 1). In addition, the general utility of the sonication technique for loading mammalian cells was demonstrated by the successful introduction of fluorescein-labeled dextran into the cytoplasm of HeLa, hepatoma, and myeloma cells (Table 1).

Cell lines exhibiting stable expression of thymidine kinase were counted after transfection of LTK^- cells with pPVTK4 and selection and growth in HAT medium as described in *Materials and Methods*. Transfection of LTK^- cells by scrape loading with pPVTK4 at 50 μg/ml in a solution containing 140 mM NaCl, glucose at 1 mg/ml, 0.1 mM EDTA, and 10 mM KH_2PO_4 at pH 7.2 resulted in an average of 19 stably transformed colonies per 10^6 cells (Table 2). No

Table 1. Loading of cells with fluorescein-labeled dextran by scrape loading or sonication loading

Cell line	Loading method	% cells loaded
Hepatoma tissue culture	Sonication	13
Mouse myeloma	Sonication	10
HeLa	Sonication	14
LTK^- fibroblast	Sonication	20
LTK^- fibroblast	Scrape	50
REF	Scrape	80

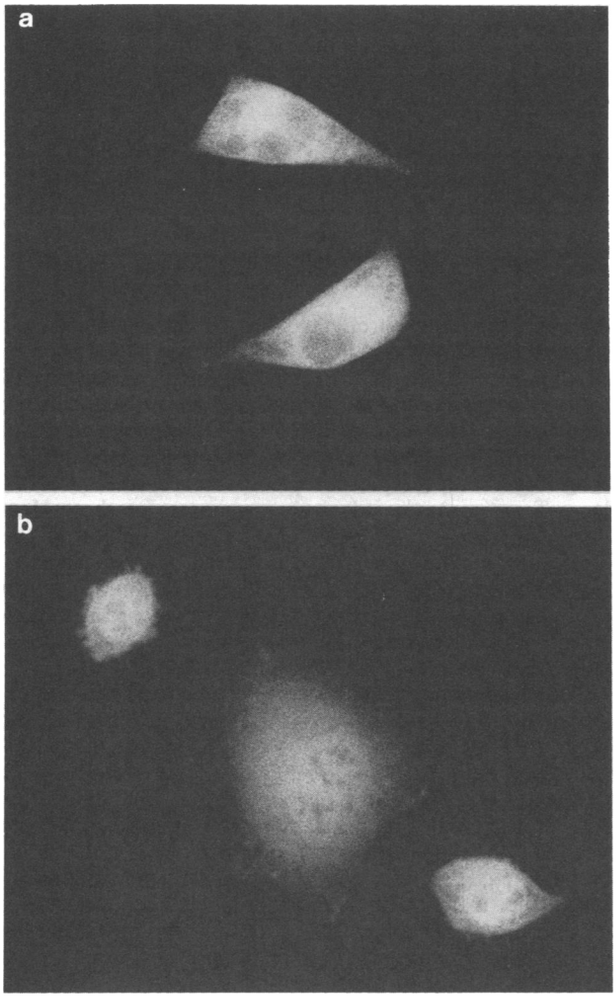

FIG. 1. Fluorescence microscopy images of LTK$^-$ fibroblasts loaded with fluorescein-labeled dextran by sonication loading (*a*) or scrape loading (*b*). (×1040.)

transformation was observed in the absence of DNA. Substitution of KCl for NaCl in the solution during scraping increased the frequency of transformants to 64 stably transformed colonies per 10^6 cells. Addition of 2 mM $MgCl_2$ caused a modest increase in transformation in the presence of NaCl, and a marked decrease in the frequency of transformants obtained in the presence of KCl (Table 2). Variation of the concentration of DNA from 10 to 50 μg/ml resulted in a rather monotonic but not strictly linear increase in the frequency of transformed colonies recovered (data not shown).

Transfection of LTK$^-$ cells by sonication loading with pPVTK4 at 50 μg/ml in a solution containing 140 mM NaCl, glucose at 1 mg/ml, 0.1 mM EDTA, and 10 mM KH_2PO_4, pH 7.2, resulted in an average of 23 transformed colonies per 10^6 cells (Table 3). No transformants were obtained when DNA was omitted. Substitution of KCl for NaCl in the loading solution increased the transformation frequency to 34 colonies per 10^6 cells. Addition of 2 mM $MgCl_2$ to the loading solutions containing NaCl and KCl caused decreases in the transformation frequency to 10 and 25 transformants per 10^6 cells, respectively.

Some colonies recovered after sonication loading were trypsinized in cloning rings and passaged repeatedly in HAT medium to demonstrate that the transfection was a stable event and could not be explained by residual enzyme transiently expressed immediately after transfection.

Transfection of REF and D2 Cells by Scrape Loading with pSV2neo. Since transfection procedures can vary with cell type, selectable marker, and the promoter driving expression of the gene, we investigated a second system for transfection by scrape loading. The system utilized is the plasmid pSV2neo (26), which contains a bacterial gene conferring resistance to neomycin and whose expression is strongly stimulated by juxtaposition with the SV40 early promoter in the plasmid construction.

REF transfected with 1 μg of pSV2neo DNA by scrape loading multiplied in the presence of Geneticin to produce colonies at a frequency of 620 foci per 10^6 cells, and 30% of these, or 186 per 10^6 cells, produced stably growing cell lines (Table 4).

We next employed the system for transfection of nonselectable markers by the method of cotransfection with a selectable marker. The pSV2neo plasmid was mixed with a plasmid encoding the gene for SV40 large T antigen (pC6), the wild-type adenovirus genome, or the cloned c-Ha-*ras* oncogene (*ras* T24) at a ratio of 1:10, and used for scrape loading. Neither the selection in the presence of Geneticin nor the growth of the cells to produce stably transfected cell lines was dramatically affected by the addition of the nonselected DNA molecules utilized. However, the cell lines isolated from cotransfections did express the nonselected marker, as assessed in the case of pC6 by immunofluorescence microscopy using antibody reactive with the SV40 large T antigen. Expression of adenovirus and *ras* T24 oncogene was verified by RNA dot blot and protein electrophoretic blotting methods. These DNA molecules transfected by the scrape loading procedure varied in size from 8000 to 30,000 base pairs (Table 4). Thus, the scrape loading cotransfection procedure is a simple method for isolation of cell lines expressing specific gene products for which the cloned genes are available.

Transient Expression of DNA Introduced by Scrape Loading. The initial cellular and biochemical changes associated with expression of a transfected gene product are best studied immediately after introduction of the DNA. The success of such experiments requires that a high frequency of the cells express the introduced gene product. We have employed

Table 2. Transfection of LTK$^-$ fibroblasts with pPVTK4 DNA by scrape loading

DNA, μg/ml	Buffer*	No. plates	Colonies per plate	Transformed colonies per 10^6 cells
0	KCl	13	0	<0.08
50	KCl	3	85, 63, 72	73
50	KCl	3	60, 53, 70	61
50	KCl	3	54, 66, 51	57
50	KCl/$MgCl_2$	2	19, 15	17
0	NaCl	2	0	<0.5
50	NaCl	2	20, 19	19
50	NaCl/$MgCl_2$	1	25	25

*The loading solution (pH 7.2) contained 10 mM KH_2PO_4, 0.1 mM EDTA, glucose at 1 mg/ml, and 140 mM either NaCl or KCl; in the indicated experiments 2 mM $MgCl_2$ was added.

Table 3. Transfection of LTK$^-$ fibroblasts with pPVTK4 DNA by sonication loading

DNA, μg/ml	Buffer*	No. plates	Colonies per plate	Transformed colonies per 10^6 cells
0	KCl	2	0	<0.5
50	KCl	2	14, 54	34
50	KCl/$MgCl_2$	3	27, 21, 19	25
50	NaCl	2	26, 19	23
50	NaCl/$MgCl_2$	4	14, 2, 8, 14	10

*The loading solutions were identical to those described for Table 2.

Table 4. Transfection and cotransfection of cells with pSV2neo

Recipient cells	pSV2neo DNA, μg	Other DNA		Size of insert, kb	Foci per 10^6 cells	% foci able to expand
		DNA	μg			
REF	1	None	—	8	620	30
REF	1	pC6	10	12	576	25
REF	2	Adenovirus 2 genome	20	30	1200	25
REF	1	*ras* T24 oncogene	10	10	500	40
D2	1	*ras* T24 oncogene	10	10	864	ND

ND, not determined.

immunofluorescence microscopy to study expression of transfected gene products between 0 and 5 days after transfection of REF with the pC6 plasmid, which encodes the SV40 large T antigen. Cytoplasmic fluorescence was observed on day 0 (5 hr after transfection). The frequency of cells containing significant quantities of the large T antigen in the nucleus rose rapidly to 60% on day 1 after transfection, and it increased slowly to 70% and 80% by day 3 and day 5 after transfection, respectively (Table 5).

To determine whether the subpopulation of cells not expressing the transfected gene product were unable to do so, or did not receive the DNA in significant quantities, we assessed the loading frequency by scrape loading these cells in the presence of fluorescein-labeled dextran. A loading frequency of approximately 80% was determined both 6 and 24 hr after scraping in two separate experiments (Table 1). Thus, nearly all cells receiving DNA by scrape loading can express the exogenous gene product.

DISCUSSION

The primary goal of the present investigation was to test the utility of scrape loading and sonication loading as tools for the expression of exogenous nucleic acid by mammalian cells. The scrape loading method had been utilized previously to introduce protein and dextran molecules into a variety of mammalian cell types (23), and sonication loading had been used to introduce such molecules into amoebae of the cellular slime mold *D. discoideum* (24, 29). Loading of fluorescein-labeled dextran into the cytosol of a number of cultured cell lines demonstrates that the sonication loading method is also applicable to mammalian cells (Fig. 1; Table 1).

The first test of sonication and scrape loading of DNA into mammalian cells was performed with the cloned thymidine kinase gene and the fibroblast cell line (LTK^-) lacking this enzyme. Growth of colonies of cells in selective medium at frequencies between 10 and 80 per 10^6 cells demonstrated that stable introduction and expression of the plasmid DNA had occurred (Tables 2 and 3). Higher frequencies of transformation were observed after both sonication loading and scrape loading when extracellular sodium was replaced with potassium in the loading solution. Addition of divalent cations had either no effect or an inhibitory effect on the number of stably transformed colonies recovered.

Table 5. Transient expression of SV40 large T antigen after transformation of REF with plasmid pC6

Time, days	% cells exhibiting nuclear T antigen	
	Exp. 1	Exp. 2
0	7	11
1	64	57
2	67	75
3	71	82
4	79	88
5	81	86

Additional studies of the introduction of exogenous DNA by the scrape loading technique have shown that this technique can be used for cotransfection experiments. A mixture of the plasmid pSV2neo, which encodes resistance to the drug G418, with the plasmid pC6 (which encodes the SV40 large T antigen), the adenovirus genome, or the c-Ha-*ras* oncogene resulted in growth of clones expressing these molecules in addition to the selected marker conferring resistance to G418. The scrape loading technique is quite useful for obtaining expression of genes whose introduction is not selectable, since the frequency of cotransfection is quite high when this loading method is used.

The scrape loading method can also be used for studies of transient expression of introduced gene products at short times after loading. As many as 80% of scrape loaded cells expressed the introduced SV40 large T gene product. This frequency is sufficiently high to permit biochemical and cellular analyses of the immediate consequences of expression of the exogenous gene product. The frequency of transient expression is nearly identical to the frequency of cells scrape loaded with fluorescein-labeled dextran, suggesting that all cells receiving DNA carry out transient expression under these conditions. This result is consistent with the report that a large proportion of cells directly microinjected with plasmid DNA exhibit transient expression of the gene product (6). In addition, J. G. Woodward (University of Kentucky) has observed expression of β-galactosidase in L-929 cells 1–2 days after scrape loading with the plasmid pON1, which contains this gene (personal communication).

Comparison of scrape and sonication loading with some of the other techniques currently in use reveals a number of potential advantages. Both methods are rapid, inexpensive to perform, and efficient. These techniques have an advantage over direct microinjection in that these methods are less labor intensive, and they offer potential either for the isolation of large numbers of transformed colonies or for preparation of large numbers of cells transiently expressing an exogenous gene product to permit extensive kinetic and biochemical studies. A second advantage of scrape and sonication loading is that the DNA may be presented in solution. Weintraub and colleagues (31) have shown that both linear and supercoiled forms of plasmid DNA differ from simple circular conformations in transformation efficiency. The conformation of the DNA could be more completely controlled if the DNA were present in soluble form, allowing more detailed studies of the effects of DNA conformation, including analyses of transformation using native DNA–protein complexes. Third, these loading techniques are quite gentle, and produce loaded cells that remain competent to grow. Thus, these techniques may be effective on cell types that have proven difficult to transform by using the existing methods. A final advantage of the scrape and sonication loading techniques is that there is no requirement to introduce the DNA into an intermediate carrier such as a liposome or erythrocyte or a biological carrier such as a retrovirus vector or *Agrobacterium*. The DNA may be added directly to the medium surrounding the

cells. Thus, the potential contribution of these loading methods to future studies of the introduction of exogenous DNA to living cells is apparent and warrants both additional investigation and immediate application.

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